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THE FERMENTATION OF D-ALLOSE AND D-GLUCOSE BY *AEROBACTER AEROGENES*[1]

BY H. A. ALTERMATT[2], F. J. SIMPSON, AND A. C. NEISH

Abstract

Aerobacter aerogenes rapidly ferments D-allose-1-C^{14} and D-allose-2-C^{14} under aerobic and anaerobic conditions to give products labelled in the same manner as those obtained from similar fermentations of D-glucose-1-C^{14} and D-glucose-2-C^{14}. The lactic acid, acetic acid, ethanol, and 2,3-butanediol obtained from the sugars labelled in carbon-1 contained C^{14} in the methyl groups. From the sugars labelled in carbon-2, *A. aerogenes* produced lactic acid, ethanol, and 2,3-butanediol labelled in the carbinol groups and acetic acid labelled in the carboxyl group. The results agree with the hypothesis that both sugars are fermented under anaerobic conditions by the Embden–Meyerhof–Parnas route. This route is also of major importance under aerobic conditions where little sugar appears to be dissimilated via the hexose monophosphate shunt.

Introduction

Aerobacter aerogenes ferments D-allose rapidly with the production of acid and gas. Since allose and ribose are closely related, the distribution of C^{14} found in the products from the anaerobic and aerobic fermentation of allose-1-C^{14} and allose-2-C^{14} was compared with that from the fermentation of glucose-1-C^{14} and glucose-2-C^{14} and that previously obtained from pentoses (1, 10) to determine if allose was dissimilated via the hexose monophosphate shunt mechanism or via the Embden–Meyerhof–Parnas route. The latter route is dominant under both aerobic and anaerobic conditions.

Materials and Methods

Preparation of Substrates

To prepare allose-1-C^{14}, D-ribose was allowed to react with an equimolar amount of $KC^{14}N$ in the presence of bicarbonate buffer and then the resultant cyanohydrins were hydrolyzed (9). Altronic acid was removed as the calcium salt, and the allonic lactone crystallized from the concentrated deionized

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[2] *National Research Council of Canada Postdoctorate Fellow, 1953-55.*

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mother liquors. The allonic lactone was reduced with 5% sodium amalgam to allose-1-C^{14} (3). Similarly allose-2-C^{14} was synthesized from ribose-1-C^{14} using non-radioactive sodium cyanide (8). Glucose-1-C^{14} was prepared by the addition of $KC^{14}N$ to D-arabinose, and glucose-2-C^{14} by the addition of cyanide to D-arabinose-1-C^{14} (3, 8, 9).

Fermentation

A. aerogenes, PRL R3 (NRRL 199), was grown and the cells harvested as previously described (10). Media for the production of cells for subsequent fermentations of labelled allose contained 0.25% allose and similarly that for subsequent fermentations of labelled glucose, 0.5% glucose. The cells were suspended in 12 ml. of a sterile mixture containing 2.5% $MgNH_4PO_4 \cdot 6H_2O$, 0.25% K_2SO_4, and 0.002% phenol red. Ten milliliters of this suspension was transferred to a modified Erlenmeyer flask of 500 ml. capacity (7) and to this was added 0.5 gm. of the radioactive sugar dissolved in 5 ml. of water. For aerobic conditions the pressure in the flasks was reduced to 0.9 atmospheres. For anaerobic conditions the air was replaced with purified nitrogen and the pressure in the flask likewise adjusted to 0.9 atmospheres. The pH was manually controlled between 7–7.8 by periodic addition of 0.03 to 0.1 ml. of *N* sodium hydroxide (10).

Examination of Products

The products were measured, isolated, and degraded to determine the position of C^{14} in the same manner as described in previous reports (7, 9).

Results

The quantities of products obtained from the fermentation of allose and glucose by *A. aerogenes* under anaerobic and aerobic conditions are given in Table I. Although *A. aerogenes* produced less 2,3-butanediol and more ethanol and acetic acid from allose than from glucose, the differences are not striking. In addition, the distribution of C^{14} among the products obtained from fermenting allose and glucose under parallel conditions is similar (Tables II, III). With both allose and glucose labelled in either carbon-1 or -2 the carbon dioxide produced under aerobic conditions contains more C^{14} than that under anaerobic conditions.

Discussion

The production of more acetic acid and ethanol, and less 2,3-butanediol from allose than from glucose (Table I) may not occur under conditions more favorable for the production of 2,3-butanediol (5). These differences may be caused by quantitative differences in the enzymic constitution of the cells and by variations in the pH of the fermentations (5, 10).

The Embden–Meyerhof–Parnas scheme for dissimilation of hexoses to lactic acid predicts that the C^{14} in carbon-1 of the hexose will be found in the methyl carbon of lactic acid and that in carbon-2 will be found in the carbinol

TABLE I

PRODUCTS OBTAINED FROM THE DISSIMILATION OF SUGARS BY *A. aerogenes*

	mM. of product per 100 mM. of sugar fermented							
	1-C^{14}-hexose				2-C^{14}-hexose			
	Anaerobic		Aerobic		Anaerobic		Aerobic	
Product	Allose	Glucose	Allose	Glucose	Allose	Glucose	Allose	Glucose
2,3-Butanediol	2.8	19.2	6.4	17.9	12.8	27.0	15.8	26.5
Ethanol	60.1	51.5	31.8	37.8	54.7	48.4	28.6	15.8
Acetic acid	67.5	51.9	61.0	48.7	68.9	40.7	54.8	55.3
Lactic acid	11.9	10.1	12.5	18.7	7.4	6.6	10.5	6.1
Formic acid	59.5	68.4	41.9	57.7	63.6	60.8	63.6	50.1
Carbon dioxide	90.0	79.6	142.9	104.9	77.4	74.9	74.0	105.1
Succinic acid	5.7	13.1	1.8	5.7	8.4.	7.5	4.4	5.9
Glycerol	2.3	4.2	1.2	—	—	—	—	—
Fermentation time (hr.)	4.5	6.0	5.0	5.5	5.0	4.0	5.0	4.0
Sugar dissimilated (%)	100.0	100.0	100.0	98.2	100.0	100.0	99.0	100.0
C^{14} in cells*	11.2	7.5	17.9	3.7	7.5	7.5	10.6	8.4
Sugar carbon accounted for (%)	91.4	95.3	94.0	84.7	90.0	86.1	80.1	82.6

* *As per cent of C^{14} added in the sugar.*

NOTE: *H_2 not measured so O/R index not calculated for anaerobic fermentations.*

TABLE II

DISTRIBUTION OF C^{14} IN PRODUCTS FROM FERMENTATION OF 1-C^{14} SUGARS

	Concentration of C^{14}, % of that in labelled carbon of hexose			
	Anaerobic		Aerobic	
Product	Allose-1-C^{14}	Glucose-1-C^{14}	Allose-1-C^{14}	Glucose-1-C^{14}
2,3-Butanediol				
CH_3—	34.7*	38.7	24.9*	31.3
—CHOH—	0.2*	0.0	0.6*	0.2
Ethanol				
CH_3—	47.6	37.1	30.5	42.2
—CH_2OH	0.2	0.2	0.5	0.2
Acetic acid				
CH_3—	42.8	44.8	45.2	46.0
—COOH	0.1	0.4	0.8	0.5
Lactic acid				
CH_3—	46.6†	37.1†	42.9†	33.0†
—CHOH—	0.2†	0.0†	0.5†	0.2†
—COOH	0.7†	1.3†	1.0†	1.0†
Formic acid	0.8	1.1	1.7	1.1
Carbon dioxide	0.5	2.9	6.3	8.2
Succinic acid	17.2	12.9	33.5	13.0
Glycerol	6.6	1.0	35.6	—

* *Diluted 10 times with carrier.*
† *Diluted five times with carrier.*

TABLE III

DISTRIBUTION OF C^{14} IN PRODUCTS FROM FERMENTATION OF 2-C^{14} SUGARS

	Concentration of C^{14}, % of that in labelled carbon of hexose			
	Anaerobic		Aerobic	
Product	Allose-2-C^{14}	Glucose-2-C^{14}	Allose-2-C^{14}	Glucose-2-C^{14}
2,3-Butanediol				
CH_3—	1.6*	0.9	1.7	1.2
—CHOH—	36.7*	37.2	36.0	36.8
Ethanol				
CH_3—	1.8	1.0	2.0	1.5
—CH_2OH	36.3	39.6	40.4	39.2
Acetic acid				
CH_3—	1.8	0.9	2.4	1.5
—COOH	41.9	41.4	41.9	40.5
Lactic acid				
CH_3—	1.7*	0.9*	2.2*	0.9*
—CHOH—	40.0*	33.5*	36.6*	22.6*
—COOH	0.7*	0.7*	1.1*	0.7*
Formic acid	0.7	0.8	0.8	1.0
Carbon dioxide	0.5	0.6	4.8	3.8
Succinic acid	11.4	9.6	10.7	10.0

* *Diluted five times with carrier.*

carbon of lactic acid. This is what was found in the anaerobic dissimilation of both allose- and glucose-1-C^{14} (Table II) and allose- and glucose-2-C^{14} (Table III). The distribution of C^{14} in the other fermentation products is consistent with the view that they were derived from pyruvate labelled in the same manner as lactic acid. The results agree with those previously obtained in the anaerobic fermentation of glucose-1-C^{14} by *Bacillus subtilis* and *A. aerogenes* (7, 10).

The Embden–Meyerhof–Parnas scheme is also the major route of dissimilation when allose and glucose are fermented under aerobic conditions (Tables II, III). The distribution of C^{14} in the products is similar to that found under anaerobic conditions. However, under anaerobic conditions the carboxyl group of lactic acid, formic acid, and carbon dioxide contain the same amount of C^{14} whereas under aerobic conditions the carbon dioxide contains more C^{14}. Thus the additional C^{14} is not obtained directly from pyruvate or formate, but may be partly derived from the citric acid cycle and partly from the hexose–pentose–heptulose cycle (1, 4, 9, 11). If the citric acid cycle was solely responsible, the carbon dioxide produced from hexose-1-C^{14} should contain about the same amount of C^{14} as that from hexose-2-C^{14}. Since approximately twice as much $C^{14}O_2$ was produced from hexose-1-C^{14} as from hexose-2-C^{14} the hexose–pentose–heptulose cycle was probably operating. A more precise estimate of the amount of hexose diverted through this pathway could be obtained from the fermentation of hexose-1- and -6-C^{14}.

Although this work shows that allose and glucose are fermented by a common pathway, it is not known how allose is incorporated into the Embden–Meyerhof–Parnas route. Allose may be phosphorylated by the same kinase as glucose since Sols and Crane found that brain hexokinase phosphorylates allose at a rate half that for glucose (12). On the other hand, the hexokinases of *Escherichia coli* appear to be quite specific (2) and perhaps the kinases of *A. aerogenes* are likewise more specific than brain hexokinase. In addition, *A. aerogenes* probably has a system for inverting the position of the hydroxyl at carbon-3 of D-allose to produce a configuration more susceptible to attack by aldolase (13). This inversion may be catalyzed by a waldenase similar to that known to attack galactose (6) or the inversion may occur via a 2,3-enediol to fructose. The explanation of how allose enters the Embden–Meyerhof–Parnas pathway will require enzymatic studies.

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References

1. ALTERMATT, H. A., SIMPSON, F. J., and NEISH, A. C. The anaerobic dissimilation of D-ribose-1-C^{14}, D-xylose-1-C^{14}, D-xylose-2-C^{14}, and D-xylose-5-C^{14} by *Aerobacter aerogenes*. Can. J. Biochem. Physiol. In press. 1955.
2. CARDINI, C. E. The hexokinases of *Escherichia coli*. Enzymologia, 14 : 362-368. 1950-51.

3. ISBELL, H. S., KARABINOS, J. V., FRUSH, H. L., HOLT, N. B., SCHWEBEL, A., and GALKOWSKI, T. T. Synthesis of D-glucose-1-C^{14} and D-mannose-1-C^{14}. J. Research Natl. Bur. Standards, 48 : 163-171. 1952.

4. KINOSHITA, J. H. and MASURAT, T. The direct oxidative carbohydrate cycle of bovine corneal epithelium. Arch. Biochem. and Biophys. 53 : 9-19. 1954.

5. LEDINGHAM, G. A. and NEISH, A. C. Fermentative production of 2,3-butanediol. *In* Industrial fermentations. Vol. II. *Edited by* L. A. Underkofler and R. J. Hickey. The Chemical Publishing Co., Inc., New York, N.Y. 1954. pp. 27-93.

6. LELOIR, L. F. The metabolism of hexosephosphates. *In* Phosphorus metabolism. Vol. I. *Edited by* W. D. McElroy and B. Glass. The Johns Hopkins Press, Balitimore, Md. 1951. pp. 67-93.

7. NEISH, A. C. Studies on the anaerobic dissimilation of glucose by *Bacillus subtilis* (Ford's type). Can. J. Botany, 31 : 265-276. 1953.

8. NEISH, A. C. Preparation of 1-C^{14} pentonic acids by the cyanhydrin synthesis. Can. J. Chem. 32 : 334-339. 1954.

9. NEISH, A. C. and BLACKWOOD, A. C. The anaerobic dissimilation of sedoheptulose-2-C^{14} and sedoheptulose-3-C^{14} by *Aerobacter aerogenes*. Can. J. Biochem. Physiol. 33 : 323-331. 1955.

10. NEISH, A. C. and SIMPSON, F. J. The anaerobic dissimilation of D-glucose-1-C^{14}, D-arabinose-1-C^{14}, and L-arabinose-1-C^{14} by *Aerobacter aerogenes*. Can. J. Biochem. Physiol. 32 : 147-153. 1954.

11. RACKER, E., DE LA HABA, G., and LEDER, I. G. Transketolase-catalyzed utilization of fructose-6-phosphate and its significance in a glucose-6-phosphate oxidation cycle. Arch. Biochem. and Biophys. 48 : 238-240. 1954.

12. SOLS, A. and CRANE, R. K. Substrate specificity of brain hexokinase. J. Biol. Chem. 210 : 581-595. 1954.

13. TUNG, T., LING, K., BYRNE, W. L., and LARDY, H. A. Substrate specificity of muscle aldolase. Biochim. et Biophys. Acta, 14 : 488-494. 1954.

YELLOW CHROMOGENIC BACTERIA ON WHEAT

II. DETERMINATIVE STUDIES[1]

By Norman James

Abstract

The predominant type of yellow chromogenic bacteria easily isolated from normal wheat and other seeds, and in the early literature referred to as *Bacterium herbicola aureum* Düggeli, is like *Xanthomonas translucens* in many aspects—in morphology, colony characteristics, type of pigment, and habitat. These bacteria differ from the latter species in that they are not pathogenic and they grow moderately in a medium containing asparagine as the sole source of carbon and nitrogen. The problem of nomenclature of these bacteria is considered. Evidence that justifies acceptance of *Pseudomonas trifolii* Huss as the legitimate name of the species and transference of the species to the genus *Xanthomonas* is presented. The name *Xanthomonas trifolii* (Huss) comb. nov. is proposed.

Introduction

Although several reports on yellow-pigmented bacteria on plants have appeared in the literature, these bacteria have received only slight consideration. As early as 1888, Beijerinck (3) proposed the name *Bacillus agglomerans* for a motile diplococcus-like rod isolated from red clover. It was weakly gelatine-liquefying and produced brownish colonies resembling those of *Proteus* spp. Winkler (27) isolated bacteria producing yellow colonies on gelatine from plum leaves and, without describing the species, named it *Bacillus mesentericus aureus*. Burri (9) considered yellow chromogenic bacteria to be the predominant bacteria on plants, often appearing in pure culture. Düggeli (13) was the first to describe these bacteria adequately. He proposed the name *Bacterium herbicola aureum*. They were present on a variety of seeds and represented 97 to 100% of the flora on five samples of wheat. Huss (15) proposed the name *Pseudomonas trifolii* for yellow-pigmented polar flagellated bacteria isolated from clover and from oat seeds. Mack (20), indicating preference for Düggeli's epithet, proposed the name *Flavobacterium herbicola*. This name was used by Bojko (5); whereas Kretovich and Rautenshtein (18) later used *Bacterium herbicola*. These bacteria were found in large numbers on wheat passing through the Winnipeg market (16); and on wheat of different varieties produced in different areas of the prairie provinces of Canada under different soil and climatic conditions (17).

A detailed study of the yellow chromogenic bacteria of wheat was undertaken because of: (*a*) the probable relationship of these bacteria to quality in wheat or products made from it, (*b*) the suggested antagonistic relationship between bacteria normally found on wheat seeds and certain soil and seed borne pathogenic fungi (10, 22), and (*c*) the close resemblance between these bacteria and *Xanthomonas translucens*, the etiological agent of the disease bacterial black chaff of cereals.

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Contribution from the Department of Microbiology, the University of Manitoba, Winnipeg, Manitoba, with financial assistance from the National Research Council of Canada.

Materials and Methods

The isolates used in this study were obtained from wheat, barley, oats, soybeans and flax passing through the Winnipeg market, or grown on the university farm. The procedure for isolating has been outlined (16). Unless otherwise stated, all studies were carried out according to procedures in the *Manual of Methods for Pure Culture Study of Bacteria* (11). For purposes of comparison, at the same time and under the same conditions studies were conducted on: *Xanthomonas translucens* f. sp. *undulosa*, strains 1552 and 3258; *X. translucens* f. sp. *cerealis*, strain 3040; *X. translucens* f. sp. *hordei-avenae*, strain 1907; *X. carotae; X. campestris;* and *X. pruni.*

Results

Morphology

Cultured on nutrient agar at 25° C. for 24 hr., the organisms were rod to coccoid forms, about 0.7μ in diameter and 0.7 to 2.5μ in length, mostly singles, and Gram-negative. In nutrient broth cultures they were actively motile. Most cells had one polar flagellum, but a few had two and a few four flagella near one pole. On the basis of morphology, cultures of the yellow chromogen and of the pathogens were the same. In Bergey's Manual (6) the length of *Xanthomonas translucens* is given as 1 to 2.5μ and of *X. campestris* as 0.7 to 2.0μ; and in about one third of the species listed the type of flagellation is other than that of a single polar flagellum.

Cultural Characteristics

Cultured on nutrient agar at 25° C. for five days, surface colonies were circular, entire, convex, amorphous, glistening, yellow, and 1–6 mm. in diameter. Platings from a small colony produced colonies in the same size range, indicating that within these limits the size of a colony was not an inherited characteristic. The color intensity was not the same in different colonies, and without exception plating from a colony of one intensity produced colonies of a wide range of intensity. Bamberg (2) referred to this color phenomenon in relation to five cultures of *Bacterium translucens* var. *undulosa* (now *Xanthomonas translucens* f. sp. *undulosa*). In other aspects likewise, the characteristics of the yellow chromogen and the pathogens were not distinguishable. In nutrient broth cultured at 25° C. both groups of cultures became turbid with a fragile pellicle, but the yellow chromogen became turbid more rapidly than did the pathogens.

Gelatine was liquefied slowly, not completely at two weeks. Litmus milk became alkaline slowly and at one month or more was peptonized.

Physiology

Sixty-three of 66 isolates of the yellow chromogen reduced nitrates to nitrites. None produced indole when cultured in tryptone broth. Two other cultures known to produce indole gave positive results when tested under the same conditions. Hydrogen sulphide was produced by all. None

hydrolyzed starch. Six typical cultures showed lipolytic action on linseed oil, triproprionin, and tributyrin when tested by the method of Hammer and Collins (14).

Dowson (12) regarded biochemical reactions in maltose, lactose, and salicin important in revealing generic relations in plant pathogens. Accordingly, each of 13 isolates of the yellow chromogen and of the seven pathogens was grown in the basal medium of Ayers, Rupp, and Johnson (1) plus 1% of the carbohydrate, and at seven days tested electrometrically for change in pH. The data are presented in Table I. Most isolates of the yellow chromogen showed closer agreement in these fermentation characteristics with *X. translucens* than did *X. carotae*, *X. campestris*, or *X. pruni*.

TABLE I

PRODUCTION OF ACID IN THE MEDIUM OF AYERS, RUPP, AND JOHNSON PLUS 1% OF THE SUGAR. FIGURES REPRESENT REDUCTION IN pH OF THE CULTURE FROM THE ARITHMETIC MEAN OF SIX CONTROLS FOR EACH SUGAR

Culture		Maltose	Lactose	Salicin
Yellow chromogen	1	2.14	0.14	1.04
Yellow chromogen	2	2.17	0.14	1.55
Yellow chromogen	3	2.12	0.14	1.35
Yellow chromogen	4	2.12	1.39	1.66
Yellow chromogen	5	2.12	0.08	1.61
Yellow chromogen	6	2.17	0.08	1.10
Yellow chromogen	7	2.16	0.04	1.54
Yellow chromogen	8	2.20	0.04	1.77
Yellow chromogen	9	2.22	0.22	1.59
Yellow chromogen	10	2.21	0.19	1.75
Yellow chromogen	11	2.21	0.16	2.01
Yellow chromogen	12	2.10	0.08	1.69
Yellow chromogen	13	1.37	0.12	1.08
X. translucens	1552	1.27	0.11	0.97
X. translucens	3258	2.17	0.25	1.23
X. translucens	3040	1.34	0.29	0.85
X. translucens	1907	1.70	0.23	0.14
X. carotae		0.08	0.27	0.08
X. campestris		0.16	0.01	0.32
X. pruni		0.10	0.10	0.13

Thirty of 31 isolates of the yellow chromogen tested in the medium of Starr and Weiss (26) showed moderate growth with asparagine as the sole source of carbon and nitogen. None of the pathogens grew. The results are presented in Table II. The isolates produced denser growth in Starr's (25) glutamic acid medium, yielding density unit values from 0.52 to 0.95 compared to the lower values shown in Table II for growth in the asparagine medium. None of the 31 isolates, nor of the pathogens tested, grew in the basal medium of Starr and Weiss (26) plus 0.1% arginine monohydrochloride. Likewise none grew in the medium of Lewis (19) containing tartaric acid as the sole source of carbon.

TABLE II

GROWTH IN THE MEDIUM OF STARR AND WEISS, WITH 0.5% ASPARAGINE AS THE SOLE SOURCE OF CARBON AND NITROGEN. FIGURES REPRESENT DENSITY UNITS CALCULATED FROM $D = 2 - \log T$, WHERE D IS DENSITY AND T IS PERCENTAGE OF LIGHT TRANSMISSION

Culture		Duplicate 1	Duplicate 2	Culture		Duplicate 1	Duplicate 2
Yellow chromogen	1	0.44	0.35	Yellow chromogen	17	0.32	0.27
Yellow chromogen	2	0.55	0.57	Yellow chromogen	18	0.38	0.35
Yellow chromogen	3	0.40	0.36	Yellow chromogen	19	0.34	0.35
Yellow chromogen	4	0.35	0.39	Yellow chromogen	20	0.27	0.36
Yellow chromogen	5	0.34	0.30	Yellow chromogen	21	0.35	0.31
Yellow chromogen	6	0.30	0.38	Yellow chromogen	22	0.35	0.32
Yellow chromogen	7	0.31	0.27	Yellow chromogen	23	0.29	0.30
Yellow chromogen	8	0.30	0.29	Yellow chromogen	24	0.36	0.34
Yellow chromogen	9	0.00	0.00	Yellow chromogen	25	0.27	0.34
Yellow chromogen	10	0.34	0.33	Yellow chromogen	26	0.37	0.37
Yellow chromogen	11	0.24	0.30	Yellow chromogen	27	0.44	0.46
Yellow chromogen	12	0.12	0.11	Yellow chromogen	28	0.29	0.30
Yellow chromogen	13	0.38	0.34	Yellow chromogen	29	0.32	0.38
Yellow chromogen	14	0.30	0.25	Yellow chromogen	30	0.28	0.30
Yellow chromogen	15	0.32	0.25	Yellow chromogen	31	0.39	0.37
Yellow chromogen	16	0.29	0.31				
X. translucens	1552	0.00	0.00	*X. carotae*		0.01	0.02
X. translucens	3258	0.00	0.00	*X. campestris*		0.01	0.00
X. translucens	3040	0.00	0.01	*X. pruni*		0.01	0.00
X. translucens	1907	0.00	0.01				

Lewis (19) reported that certain pathogens did not grow in this medium. Burkholder (7) grew representatives of *Phytomonas* (*Xanthomonas*) *campestris* group in a medium with inorganic nitrogen as the sole source of nitrogen. This would indicate that the nitrogen supply was adequate in the medium used for testing tartaric acid as the sole source of carbon. Burkholder (8) reported that certain species of *Xanthomonas* were acid tolerant. Twenty-four of 25 isolates of the yellow chromogen grew in nutrient broth at pH 4.5. Only one of the seven pathogens grew. The isolate that differed from the others in this respect was the one that differed in relation to the use of asparagine and one of the three that differed in the reduction of nitrates.

Pigments

The pigments of the isolates, and of the pathogens also, reacted positively to the lipocyan test and were considered to be carotenoids (21). Further studies, after the procedure of Sobin and Stahly (23), failed to indicate a difference in the pigments produced by the two groups of cultures. The pigments were extractable in ethanol, methanol, and acetone; but not in petroleum ether, carbon disulphide, carbon tetrachloride, chloroform, ethyl

ether, or benzene. They were found to be carotenoid alcohols on the basis of partition tests. The methanol extract of moist cells did not show a color reaction on the addition of an acid or a base. A petroleum ether extract of the dried pigment became pale blue on the addition of concentrated H_2SO_4, but showed no color change with HCl. Chromatographic adsorption showed two pigments common to the yellow chromogen and pathogens. A third pigment occurred as often in the chromogen as in the pathogens studied. Absorption spectrum maxima obtained on a Gaertner spectroscope are presented in Table III. Pigments from *Sarcina lutea* were introduced in this study as a check on technique. Sobin and Stahly (23) reported two maxima for *S. lutea*, at 440 and 470 mμ respectively. Starr (24) reported the maximum on crude ethanol extracts of pigments from various species of *Xanthomonas* to be 445 mμ, but did not record the concentration of ethanol used.

TABLE III

ABSORPTION SPECTRUM MAXIMA IN mμ ON PIGMENTS IN 95% ETHANOL

Culture	Original[1]		Reconstituted[2]	
Yellow chromogen 1	450	—	440	470
Yellow chromogen 2	451	—	440	470
Yellow chromogen 3	450	—	441	470
Yellow chromogen 4	450	—	440	469
Yellow chromogen 5	449	—	440	470
Yellow chromogen 6	450	—	440	470
Yellow chromogen 31	450	—	440	469
X. translucens 1552	450	—	440	470
X. translucens 3258	450	—	439	470
X. translucens 3040	451	—	440	470
X. translucens 1907	449	—	440	470
X. carotae	451	—	440	470
X. campestris	450	—	441	470
X. pruni	440	470	440	470
Sarcina lutea	439	469	440	471

[1] *Extract from moist cells.*
[2] *Eluted from adsorption column with 95% ethanol.*

Pathogenicity

Tests for pathogenicity were conducted with 25 isolates on Thatcher wheat, Victory oats, and Titan barley. Not one of 500 tests gave characteristic lesions of disease, whereas in every case the pathogens tested under the same conditions produced dark water-soaked areas around the site of infection in susceptible host plants for each of the four *formae speciales* of *X. translucens* tested.

Discussion

There is little doubt that the yellow chromogen referred to in this report is the same as that described by Düggeli (13) and by Huss (15) and studied in more detail by Mack (20). The last-named investigator expressed a similar opinion about the isolates she studied and those referred to in separate papers by the two other investigators mentioned above. Unfortunately, there is not unanimity about the nomenclature of the species; and the problem is complicated by lack of adequate description in some cases and failure to apply accepted rules of nomenclature in others. Beijerinck (3) at an earlier date assigned the binomial *Bacillus agglomerans* to a species inadequately described, and undoubtedly not the same as that referred to by Düggeli, or Huss, or Mack. Beijerinck's organism, isolated from red clover, was a weakly gelatine-liquefying, motile, brownish diplococcus-like saprophytic bacillus producing colonies resembling those of *Proteus*. This binomial, accordingly, does not warrant further consideration. Unfortunately, Düggeli used an illegitimate trinomial *Bacterium herbicola aureum* to replace the illegitimate *Bacillus mesentericus aureus* Winkler (27), which he considered to be identical with the organism he proceeded to describe. He claimed that Winkler's description was inadequate and that the name was misleading, since the organism had nothing in common with the potato bacillus. Düggeli repeatedly referred to the species and made no statement that could be interpreted as indicating that the third part of the trinomial designated a variety or subspecies of *Bacterium herbicola*. In fact, he described a second species and named it *Bacterium herbicola rubrum*—another illegitimate trinomial which he designated as a new species. The evidence is clear that Düggeli failed to recognize the need for a binomial as a basic principle of nomenclature. Nor can the name *Bacillus herbicola* used by Beijerinck (4) in reference to Düggeli's organism be considered validly published, since it was cited merely as a synonym for *Bacillus agglomerans* for which he claimed priority. The name *Pseudomonas trifolii* Huss (15), therefore, can be accepted as the first effectively published and valid binomial for the species. Mack (20) later accepted *Bacterium herbicola* Düggeli, evidently without concern about dropping the third word of the trinomial, and proposed the name *Flavobacterium herbicola* comb. nov. In the opinion of the present author, this was an illegitimate homonym, since the effectively published *Pseudomonas trifolii* Huss had priority. Others (5, 18) likewise used the epithet *herbicola*, evidently on the assumption that its validity had been established.

Likewise, there can be little doubt that this species is sufficiently like *Xanthomonas translucens* to warrant inclusion in the genus. The name *Xanthomonas trifolii* (Huss) comb. nov. is proposed, notwithstanding the fact that in Bergey's Manual (6) inability to use asparagine as the sole source of carbon and nitrogen is included in the characteristics of *Xanthomonas*. Dowson (12) did not mention asparagine in the original report on this genus, and such an arbitrary dividing line cannot place in separate genera two species that are alike in so many aspects.

Acknowledgments

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References

1. AYERS, S. H., RUPP, P., and JOHNSON, W. T. A study of the alkali forming bacteria in milk. U.S. Dept. Agr. Bull. 782. 1919.
2. BAMBERG, R. H. Black chaff disease of wheat. J. Agr. Research, 52 : 397-417. 1936.
3. BEIJERINCK, M. W. Die Bakterien der Papillionaceen-Knöllchen. Bot. Ztg. 46 : 742-750. 1888.
4. BEIJERINCK, M. W. and RANT, A. Wundreiz, Parasitismus und Gummifluss bei den Amygdaleen. Zentr. Bakteriol. Parasitenk. Abt. II, 15 : 336-375. 1906.
5. BOJKO, E. Über des Dunkelwerden des Roggenkorns bei der Lagerung. Zentr. Bakteriol. Parasitenk. Abt. II, 99 : 340-341. 1938.
6. BREED, R. S., MURRAY, E. G. D., and HITCHENS, A. P. Bergey's Manual of determinative bacteriology 6th ed. The Williams & Wilkins Company, Baltimore. 1948.
7. BURKHOLDER, W. H. The taxonomy and nomenclature of the phytopathogenic bacteria. Phytopathology, 29 : 128-136. 1939.
8. BURKHOLDER, W. H. Bacteria as plant pathogens. Ann. Rev. Microbiol. 2 : 389-412. 1948.
9. BURRI, R. Die Bakterienvegetation auf der Oberfläche normal entwickelter Pflanzen. Zentr. Bakteriol. Parasitenk. Abt. II, 10 : 756-763. 1903.
10. CHINN, S. H. F. An antibiotic producing bacterium of the genus *Pseudomonas*. Can. J. Microbiol. 1 : 118-124. 1954.
11. Committee on Bacteriological Technic of the Society of American Bacteriologists. Manual of methods for pure culture study of bacteria. Biotech. Publications, Geneva, N.Y. 1952.
12. DOWSON, W. J. On the systematic position and generic names of the Gram negative bacterial plant pathogens. Zentr. Bakteriol. Parasitenk. Abt. II, 100 : 177-193. 1939.
13. DÜGGELI, M. Die Bakterienflora gesunder Samen und daraus gezogener Keimpflänzchen. Zentr. Bakteriol. Parasitenk. Abt. II, 12 : 602-614. 1904; 13 : 56-63, 198-207. 1904.
14. HAMMER, B. W. and COLLINS, M. A. The numbers of lipolytic bacteria in various dairy products as determined with nile-blue sulphate. Iowa State Coll. Expt. Sta. Research Bull. 169 : 75-92. 1934.
15. HUSS, H. Morphologisch-physiologische Studien über zwei aroma-bildende Bacterien, *Bacillus esterificus* Maassen und *Pseudomonas trifolii* nov. sp. Zentr. Bakteriol. Parasitenk. Abt. II, 19 : 50-70, 149-161. 1907.
16. JAMES, N., WILSON, J., and STARK, E. The microflora of stored wheat. Can. J. Research, C, 24 : 224-233. 1946.
17. JAMES, N. Yellow chromogenic bacteria on wheat. I. Quantitative studies. Can. J. Microbiol. 1 : 271-274. 1955.
18. KRETOVICH, V. L. and RAUTENSHTEIN, J. J. Microbiological and biochemical processes in the self-heating of newly harvested wheat grain. Biol. Abstr. 17 : 2172. 1943.
19. LEWIS, I. M. Growth of plant pathogenic bacteria on synthetic culture media with special reference to *Phytomonas malvaceara*. Phytopathology, 20 : 723-731. 1930.
20. MACK, E. Untersuchungen über *Bacterium herbicola*. Zentr. Bakteriol. Parasitenk. Abt. II, 95 : 218-261. 1936.
21. PALMER, L. S. Carotenoids and related pigments: The chromolipoids. American Chemical Society monograph series. The Chemical Catalog Co., Inc., New York. 1922.
22. SIMMONDS, P. M. The influence of antibiosis on *Helminthosporium sativum*. Sci. Agr. 27 : 625-632. 1947.
23. SOBIN, B. and STAHLY, G. L. The isolation and absorption spectrum of bacterial carotenoid pigments. J. Bacteriol. 44 : 265-276. 1942.
24. STARR, M. P. Studies of phytopathogenic bacteria. Reprint. Abstract of Thesis, Cornell University, Ithaca, N.Y. 1943.
25. STARR, M. P. The nutrition of phytopathogenic bacteria. I. Minimal nutritive requirements of the genus *Xanthomonas*. J. Bacteriol. 51 : 131-143. 1946.
26. STARR, M. P. and WEISS, J. E. Growth of phytopathogenic bacteria in a synthetic asparagine medium. Phytopathology, 33 : 314-318. 1943.
27. WINKLER, W. Untersuchungen über das Wesen der Bakterien und deren Einordnung im Pilzsystem. Zentr. Bakteriol. Parasitenk. Abt. II, 5 : 569-579, 617-630. 1899.

THE EFFECT OF MAGNESIUM, POTASSIUM, AND IRON ON THE GROWTH AND MORPHOLOGY OF RED HALOPHILIC BACTERIA[1]

By Helen J. Brown and N. E. Gibbons[2]

Abstract

Maximum growth of the red halophilic bacteria occurs in the presence of 3.0 to 5.0 *M* sodium chloride, 0.1 to 0.5 *M* magnesium ion, 50 to 100 p.p.m. (1.3 to 2.5 × 10^{-3} *M*) potassium ion, and 0.5 to 1.0 p.p.m. (1 to 2 × 10^{-5} *M*) ferrous ion. In magnesium deficient media the rod forms become coccoid. Some strains can be adapted to grow in low concentrations of magnesium (0.01 *M*) and these adapted strains retain a coccoid form even when transferred to higher concentrations of magnesium. Growth does not occur when potassium is removed from the medium. The potassium requirement may be satisfied by rubidium but not by cesium, lithium, or ammonium ions. Potassium partially replaces the sodium chloride requirement of the red halophiles.

Introduction

The red halophiles are a group of extremely aerobic bacteria which, although covering a range of morphological types, all produce red pigmented colonies and require at least 15% sodium chloride in the medium for growth. Earlier workers cultured these organisms on complex media containing fish infusion, yeast extract, starch, or milk; however a detailed investigation of the nutritional requirements of the group has awaited the development of a chemically defined media. In 1949 Weber (23) described a synthetic medium which allowed some growth but required the addition of gelatin hydrolyzate for heavy growth. Although a high level of magnesium was said to favor growth, no data on the essential nature of this or any other constituent of the medium were included. Katznelson and Lochhead (6) found that vitamins, purines, and pyrimidines were not essential for growth of the red halophiles but that both yeast extract and its ash produced considerable stimulation.

In the present investigation the effects of magnesium, potassium, and iron on the growth and morphology of the red halophiles have been studied in some detail.

Materials and Methods

Media

The complete medium contained 1.5% casamino acids (Difco), 0.25% glutamic acid, 0.3% sodium citrate, 0.2% potassium chloride, 2.5% magnesium sulphate (heptahydrate), and 22.0% sodium chloride in distilled water (w/v). The medium was made up to four fifths of the final volume, adjusted to pH 7.2–7.7 with sodium hydroxide, and autoclaved five minutes at 120° C. The medium was then filtered to remove precipitate, the pH was adjusted to 7.0 with hydrochloric acid, and water added to give the final

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volume. Ten-milliliter aliquots were dispensed into 50-ml. Erlenmeyer flasks. Cotton plugs wrapped in cheesecloth were used except in the potassium and iron studies when the flasks were covered with 30-ml. beakers to avoid possible contamination with cotton fibers. The dispensed medium was sterilized at 120° C. for 15 min.

To study the effects of magnesium, potassium, and iron, media deficient in these ions were prepared as follows. The complete medium with magnesium sulphate omitted was sufficiently deficient in magnesium to be used without further treatment. For potassium studies, potassium chloride was omitted from the complete medium and casamino acids replaced with vitamin-free casamino acids (1%), which contained 1/10th as much potassium. The remaining traces of K^+ were removed by the biological purification method of MacLeod (9). The medium was heavily inoculated with the organism under test and incubated, with aeration, at 37° C. for four to five days. The cells were removed by centrifugation, the medium adjusted to pH 7.0 with hydrochloric acid and sterilized. For iron studies, Fe^{++} was chelated with 2,2′-bipyridine, which was added to the complete medium before sterilization (5).

Metallic ions were added to the various mineral deficient media as their reagent grade salts: K^+, Rb^+, Cs^+, Li^+, and NH_4^+ as chlorides; Mg^{++} as $MgSO_4 \cdot 7H_2O$; and Fe^{++} as $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$. To avoid formation of precipitates, Fe^{++} was added to the medium after sterilization, but all other ions were added before sterilization. Increasing amounts of magnesium sulphate cause a progressive decrease in the pH of the medium. Since the pH was difficult to readjust without precipitation, it was more convenient to adjust the pH of the broth before the first sterilization. For example, with 1.0 *M* magnesium sulphate, the pH was adjusted to about 8.2 to give a final pH of 7.0. Using this procedure little magnesium was lost and final concentrations were even slightly higher than in the original because of water losses during autoclaving. All solutions and mineral deficient media were prepared with demineralized water obtained by passing distilled water through a Filtre-Ion unit[3].

Glassware was soaked overnight in chromic acid cleaning solution, rinsed six times with tap water, six times with distilled water, and, when mineral elements other than Mg^{++} were studied, three times with demineralized water. After cleaning, iron-free glassware was prepared by filling the vessels with demineralized water and autoclaving for 30 min. just before use (18).

Cultures

Halobacterium halobium, obtained from Dr. Pijper (17), and *Pseudomonas salinaria*, *P. cutirubra*, and *Sarcina littoralis*, obtained from Dr. Lochhead (7), were selected as representative strains of red halophiles and studied most extensively. Sixteen other cultures were used in most of the tests, but, as the results supported those obtained with the representative species, they are

[3] *LaMotte Chemical Co., Towson, 4, Md.*

not reported in detail. These cultures included strains isolated by various workers from salted products, as well as strains recently isolated in this laboratory from salted casings, saline soils, and solar salts. Stock cultures were maintained on agar slants prepared by adding 2% agar to the complete medium.

Test Procedure

Cells for inocula, grown in the complete medium for three to four days at 35° C., were harvested by centrifugation, washed twice with and resuspended in iso-osmotic sodium chloride solution to give a suspension with an optical density of 0.05 at 660 mμ. The standard inoculum was 1 ml. of this suspension added to 100 ml. of medium. Larger inocula gave shorter lag periods but reduced the sensitivity of the tests. Sufficient aeration for the rod forms was obtained in the shallow layers used and only the more aerobic sarcinas required additional aeration with compressed air. In later tests, a few cultures were shaken on a rotary shaker. Growth was determined by measuring the turbidity of the cultures at 660 mμ and is expressed as optical density. Readings were taken after seven days, incubation at 35° C. for the rod forms and after 10 days for the slower growing sarcinas.

Since high salt concentrations interfere with the usual staining techniques, all morphological studies were made using phase contrast microscopy. Specimens for photography were prepared by streaking a drop of culture across a thin agar block on a slide and then lowering a cover into position to reduce movement of the cells and bring the entire length of long cells (10–20μ) into sharp focus. A long focus condenser-top-element was used with the agar block preparations. Photographs were made with a Spencer photomicrographic camera using Kodak Super Pancro-Press, Type B film and a Wratten B green filter.

Results

Effect of Magnesium on Growth

When magnesium was omitted, the medium contained very low concentrations of this ion (20 μgm./ml., 0.001 *M*) and did not support growth of any of the red halophiles tested even after prolonged incubation. Some growth was obtained at concentrations of 0.01 to 0.025 *M* (Fig. 1) but maximum growth occurred within the range 0.1 to 0.5 *M* Mg^{++}, depending on the species and amount of aeration. Shaking the cultures increased the amount of growth and reduced the optimum concentration for *P. cutirubra* from 0.25 to 0.1 *M*. A similar effect was observed for *P. salinaria* and *S. littoralis* but the optimum concentration for *H. halobium* was unchanged. In standing cultures *P. salinaria* and *H. halobium* grew fairly well in 1.0 *M* Mg^{++} initially, but died out after three or four transfers. In shake cultures the deleterious effect of high concentrations of magnesium became evident even earlier.

At optimal concentrations of magnesium, the sulphate, chloride, and nitrate were equally effective as sources of this cation but at higher concentrations the

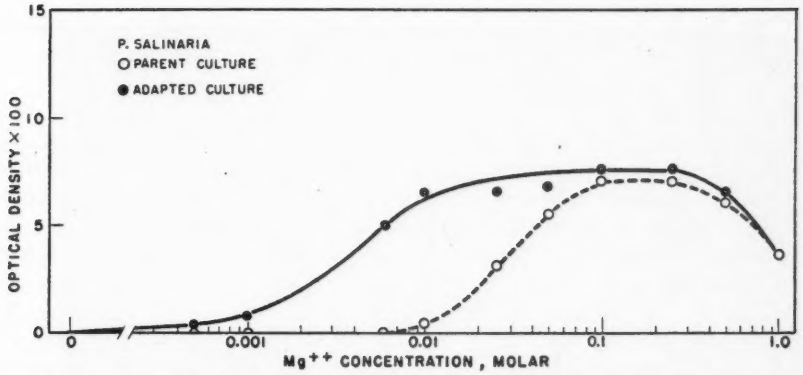


FIG. 1. Effect of concentration of magnesium on growth of *P. salinaria*, *P. cutirubra*, *H. halobium*, and *S. littoralis*. Curves marked *S* are shake cultures, all others standing cultures.

nitrate was toxic. Neither Mn^{++} nor Ca^{++} could replace Mg^{++}. The 16 additional cultures showed similar growth responses in the presence of magnesium.

Effect of Magnesium on Morphology

Magnesium had a marked effect on the morphology of *P. salinaria* and the other rod-shaped halophiles. At 0.1 *M* Mg^{++} the majority of cells were motile rods with rounded ends, 3–15μ × 0.75μ (Figs. 2 and 10). An increase in Mg^{++} to 0.25 *M* greatly increased the incidence of elongated cells, 15–30μ in length (Fig. 3), but as the culture aged only cells of normal length were seen, indicating that cell division was delayed rather than inhibited. With high magnesium concentrations (1.0 *M*) the cells became shorter and there was no apparent interference with cell division (Fig. 4). At very low levels (0.01 *M*) the normal rods were completely replaced by coccoid forms, 1.5–3μ in diameter (Figs. 5 and 11), which had a sluggish tumbling motion. Appendages resembling very short flagella were often seen in wet preparations but attempts to photograph them were unsuccessful. Dividing cells, seen as diplococci, indicated that magnesium deficiency did not interfere with cell division. The coccoid forms reverted to the normal rod shape when transferred to media containing 0.1 *M* magnesium.

P. salinaria could be adapted to grow at low magnesium levels. After five transfers some reduction in the Mg^{++} requirement was evident, and after 20 transfers the adapted strain required only 1/10th as much Mg^{++} as the parent strain to reach maximum growth (Fig. 15). The morphology of the adapted strain was characteristic of magnesium deficiency (Figs. 6–8), but the coccoid cells had lost the ability to revert to rods when transferred to media containing 0.1 *M* Mg^{++}. Even after the adapted strain had been carried for six months (10 transfers) in media containing 0.1 or 0.25 *M* Mg^{++}, the cells still retained the coccoid form (Fig. 9).

No change in morphology was noted when *S. littoralis* and other halophilic cocci were grown in different concentrations of magnesium (Figs. 12–14).

Effect of Potassium

P. salinaria, *P. cutirubra*, *H. halobium*, and *S. littoralis* failed to grow in media from which the potassium had been removed. Maximum growth of the four species occurred in media containing 5–10 mgm. % K^+ (0.0013–0.0025 *M*) but concentrations up to 3 *M* (as potassium chloride) were not inhibitory. Deficiency or excess of potassium had no effect on the morphology of the organisms.

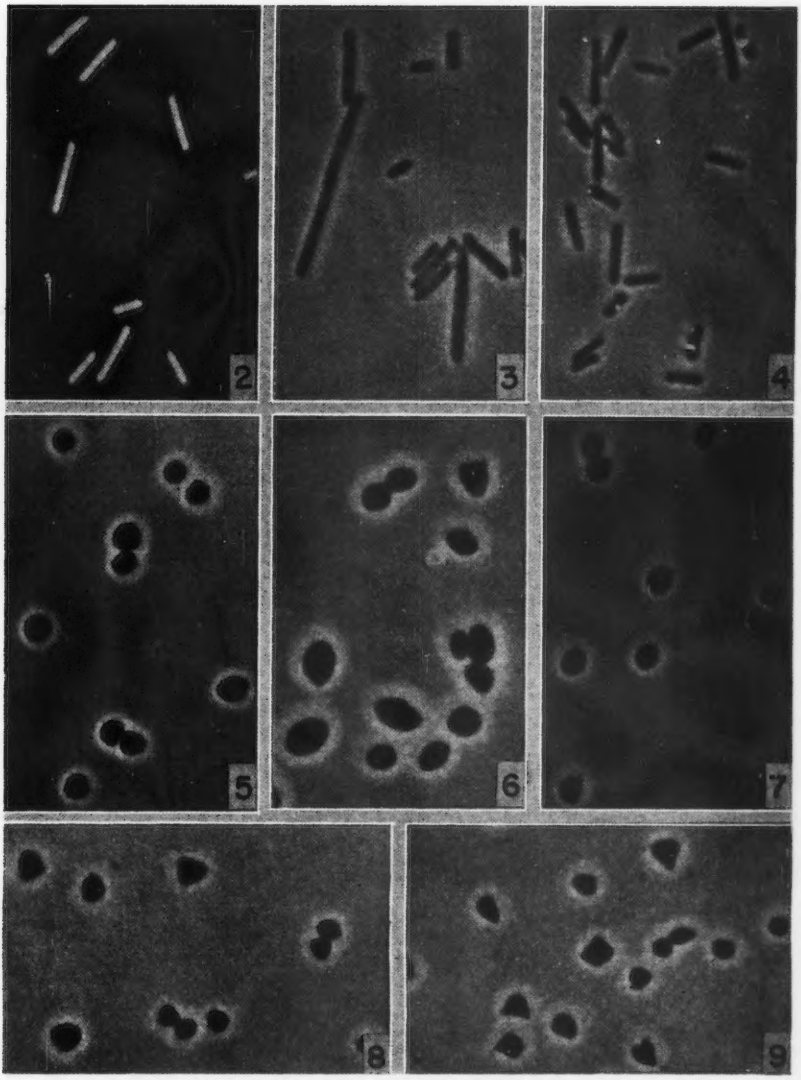
Growth of *P. salinaria*, *P. cutirubra*, and *S. littoralis* was obtained when K^+ was replaced by Rb^+ (cf. 11) but 10 to 100 times as much of the latter ion was required for equivalent growth, and the color was unusually pale suggesting that potassium may be involved in pigment production. The K^+ requirement could not be satisfied by Cs^+, Li^+, or NH_4^+.

Abundant growth of *P. salinaria* was obtained in the complete medium containing 3 to 5 *M* NaCl, but growth was absent when the sodium chloride concentration was reduced to 2.5 *M*, i.e. a NaCl deficiency of 0.5 moles. Sodium chloride deficiencies of 0.5 to 1.5 moles could be made up with KCl, although usually more KCl than NaCl was required (Table I). However, a NaCl deficiency of 2.0 moles could not be made up regardless of the concentration of potassium chloride and despite the fact that the total molarity and

TABLE I

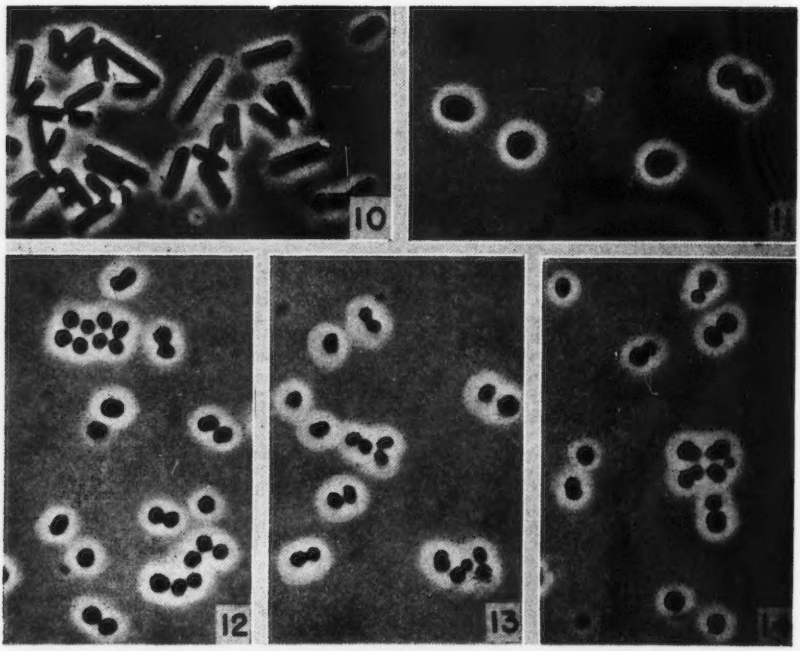
THE EFFECT OF POTASSIUM CHLORIDE ON THE SODIUM CHLORIDE REQUIREMENT OF *P. salinaria*

Concentration of NaCl (molar)	Optical density							
	Concentration of KCl (molar)							
	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5
5.0	.469	—	—	—	—	—	—	—
4.5	.432	—	—	—	—	—	—	—
4.0	.357	—	—	—	—	—	—	—
3.5	.319	—	—	—	—	—	—	—
3.0	.194	.276	.347	.367	.328	—	—	—
2.5	0	.097	.276	.357	.357	—	—	—
2.0	—	—	.013	.237	.222	.201	—	—
1.5	—	—	—	.027	.086	.208	.155	—
1.0	—	—	—	—	0	0	0	0



FIGS. 2–9. Effect of magnesium concentration on the morphology of *P. salinaria*. Phase contrast illumination, FIG. 2 light M objective, others dark M. Magnification 1700 ×. FIG. 2. Normal cells, 0.1 *M* Mg. FIG. 3. Elongated cells, one transfer in 0.25 *M* Mg. FIG. 4. Cells after one transfer in 1.0 *M* Mg. Refractive bodies are the "gas vacuoles" of Petter (14) or the "inclusion bodies" of Spruit and Pijper (17) (cf. Fig. 10). FIG. 5. Cells after one transfer in 0.01 *M* Mg. FIG. 6. Adapted strain after 15 transfers in 0.01 *M* Mg. FIGS. 7 and 8. Adapted strain after 20 transfers in 0.01 *M* Mg. FIG. 9. Adapted strain after 16 transfers in 0.01 *M* Mg. followed by 10 transfers in 0.1 *M* Mg.

PLATE II



FIGS. 10–14. Effect of magnesium on morphology of a halophilic rod and of *S. littoralis*. Phase contrast illumination. Magnification 1700 ×. FIG. 10. Halophilic rod (No. 4). Normal cells grown in 0.1 *M* Mg. FIG. 11. Cells of rod No. 4 after one transfer in 0.01 *M* Mg. FIGS. 12, 13, and 14. Cells of *S. littoralis* grown in 0.1 *M*, 0.01 *M*, and 1.0 *M* Mg respectively.

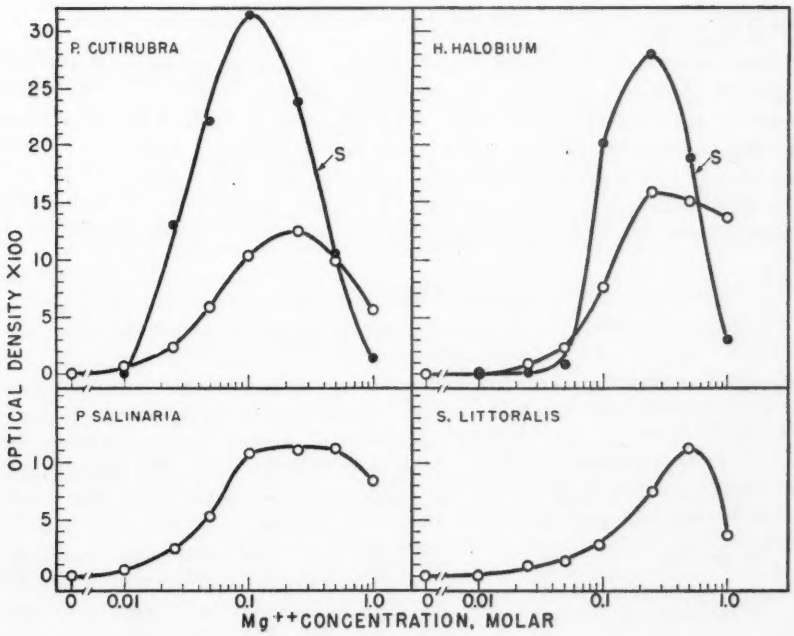


FIG. 15. Effect of magnesium concentration on growth of *P. salinaria*. Open circles—parent strain, closed circles—strain adapted to low concentration of magnesium.

ionic strength (cf. 4) was considerably in excess of that necessary to support growth with NaCl alone. Rubidium chloride was able to make up NaCl deficiencies of 0.5 and 1.0 moles, but not of 1.5 moles. Sodium chloride deficiencies of 0.5 moles, but not of 1.0 moles, could be made up by magnesium sulphate, sodium bromide, sodium nitrate, sodium sulphate, potassium nitrate, potassium sulphate, and lithium chloride. These findings also demonstrate a requirement for both Na^+ and Cl^-. Growth ceases when the sodium concentration falls below 1.5 *M*, even in the presence of large amounts of potassium. The chloride requirement can be replaced only partially by bromide, nitrate, and sulphate.

The addition of yeast extract to a potassium deficient medium stimulated growth of *P. salinaria* to a somewhat greater extent than did the ash of an equivalent weight of extract. Most of the stimulating effect of the ash was the result of its potassium content. The addition of the ash from 100 mgm. yeast extract, which analysis showed contained 3 mgm. K^+, produced almost the same amount of growth as 3 mgm. K^+ added as KCl. The slight additional effect of yeast extract may be related to its amino acid or peptide content (2), as vitamins, purines, and pyrimidines are not essential for growth of red halophiles (6).

Effect of Ferrous Ion

Good growth of all cultures was obtained in the complete medium and the addition of ferrous ion did not stimulate growth (Table II). However, growth was completely inhibited by the addition of 25 μgm. of 2,2′-bipyridine per ml. of medium, which should chelate approximately 3 μgm. of Fe^{++} per ml. (5). Reversal of this inhibition by the addition of 0.5 μgm. of Fe^{++} per ml. indicated that iron is essential. The iron requirement of halophiles (approximately 0.00001 *M*) is similar to that of other bacteria (16, 19).

TABLE II

EFFECT OF Fe^{++} ON GROWTH OF *P. salinaria*, *P. cutirubra*, AND *H. halobium* AFTER SEVEN DAYS AT 35° C.

		Optical density							
	Concentration of 2,2′-bipyridine (μgm./ml.)	Concentration of Fe^{++} (μgm./ml.)							
Culture		0	0.01	0.05	0.1	0.5	1.0	5.0	10.0
P. salinaria	0	.167	.187	—	.174	—	.180	—	.155
	25	0	.009	.022	.027	.125	.108	.149	.155
P. cutirubra	0	.131	.137	—	.131	—	.149	—	.125
	25	.009	.018	.027	.050	.114	.108	.149	.114
H. halobium	0	.125	.137	—	.119	—	.097	—	.071
	25	.009	.018	.046	.046	.102	.097	.114	.108

Discussion

Magnesium has not been reported previously as an essential element for the red halophiles, although its stimulating effect has been recognized (3, 23). The unusually high requirement of magnesium for growth and the ability of these organisms to grow in the presence of relatively large amounts of magnesium sulphate are without parallel amongst other groups of bacteria (9, 10, 13, 16, 20, 21, 22). At present no explanation can be offered for the high magnesium requirement; it is greatly in excess of the quantities required by known Mg^{++} activated enzymes and the ion does not seem to be incorporated into the cell (unpublished data).

The effect of low magnesium concentration on the morphology of the rod forms is striking. The coccoid forms observed are in contrast to the filamentous forms of clostridia and bacilli observed by Webb (20, 21) in magnesium deficient media. Furthermore, the explanation that magnesium is required for cell division does not hold since dividing cells were observed at low magnesium concentrations (cf. Fig. 5). The mechanism by which magnesium affects the shape of halophilic rods is not known. Magnesium deficiency has no effect on the morphology of halophilic cocci, an observation

in agreement with that of Webb (21) on other cocci, and the chain formation reported by Rochford and Mandle (15) for *Diplococcus pneumoniae* in magnesium deficient media was not seen.

The ability of some strains to adapt themselves to low magnesium concentrations, with the accompanying irreversible change in morphology, may have taxonomic implications. The fact that only some strains could be trained in this way, as well as the observation that freshly isolated strains required as much magnesium for maximum growth as old cultures, indicates that the initially high magnesium requirement was not the result of cultivation on artificial media.

The potassium requirement of the halophiles is only slightly higher than that reported for many other organisms (8, 10, 16) and there was no indication of antagonism between sodium and potassium (11, 12, and 16). There is some evidence that potassium is stored in the cells (unpublished data) and, since it is known that some of the enzymes of halophiles operate more efficiently in the presence of potassium than of sodium (1), potassium no doubt has an important intracellular role. At first glance, the replacement of part of the sodium chloride requirement of the halophiles by potassium appears to be a simple osmotic effect. However, potassium, sodium, and chloride must play a more complicated role since potassium chloride replaces the sodium chloride more efficiently than any of the other salts tested, and since only a portion of the sodium chloride requirement can be replaced by potassium.

References

1. BAXTER, R. M. and GIBBONS, N. E. The glycerol dehydrogenases of *Pseudomonas salinaria*, *Vibrio costicolus*, and *Escherichia coli* in relation to bacterial halophilism. Can. J. Biochem. Physiol. 32 : 206-217. 1954.
2. COLEMAN, I. W. A study, by means of paper chromatography, of the growth stimulating properties of liver extract, yeast solubles, and trypsin digest of casein for *Lactobacillus casei*. Can. J. Med. Sci. 29 : 151-158. 1951.
3. DUSSAULT, H. P. and LACHANCE, R. A. Improved medium for red halophilic bacteria from salted fish. J. Fisheries Research Board Can. 9 : 157-163. 1952.
4. FLANNERY, W. L., DOETSCH, R. N., and HANSEN, P. A. Salt desideratum of *Vibrio costicolus*, an obligate halophilic bacterium. I. Ionic replacement of sodium chloride requirement. J. Bacteriol. 64 : 713-717. 1952.
5. HICKEY, R. J. The inactivation of iron by 2,2′-bipyridine and its effect on riboflavin synthesis by *Clostridium acetobutylicum*. Arch. Biochem. 8 : 439-447. 1945.
6. KATZNELSON, H. and LOCHHEAD, A. G. Growth factor requirements of halophilic bacteria. J. Bacteriol. 64 : 97-103. 1952.
7. LOCHHEAD, A. G. Bacteriological studies on the red discoloration of salted hides. Can. J. Research, 10 : 275-286. 1934.
8. LWOFF, A. and IONESCO, H. L'ion potassium et la croissance de la bactérie *Moraxella lwoffi*. Ann. inst. Pasteur, 79 : 14-19. 1950.
9. MACLEOD, R. A. Further mineral requirements of *Streptococcus faecalis*. J. Bacteriol. 62 : 337-345. 1951.
10. MACLEOD, R. A. and SNELL, E. E. Some mineral requirements of the lactic acid bacteria. J. Biol. Chem. 170 : 351-365. 1947.
11. MACLEOD, R. A. and SNELL, E. E. The effect of related ions on the potassium requirements of lactic acid bacteria. J. Biol. Chem. 176 : 39-52. 1948.
12. MACLEOD, R. A. and SNELL, E. E. The relation of ion antagonism to the inorganic nutrition of lactic acid bacteria. J. Bacteriol. 59 : 783-792. 1950.
13. MACLEOD, R. A., ONOFREY, E., and NORRIS, M. E. Nutrition and metabolism of marine bacteria. 1. Survey of nutritional requirements. J. Bacteriol. 68 : 680-686. 1954.

14. PETTER, H. F. M. Over roode en andere bacteriën van gezouten visch. Thesis, University of Utrecht, Holland. 1932.

15. ROCHFORD, E. J. and MANDLE, R. J. The production of chains by *Diplococcus pneumoniae* in magnesium deficient media. J. Bacteriol. 66 : 554-560. 1953.

16. SHANKAR, K. and BARD, R. C. The effect of metallic ions on the growth and morphology of *Clostridium perfringens*. J. Bacteriol. 63 : 279-290. 1952.

17. SPRUIT, C. J. P. and PIJPER, A. An obligate halophilic bacterium from solar salt. Antonie van Leeuwenhoek. J. microbiol. serol. 18 : 190-200. 1952.

18. WARING, W. S. and WERKMAN, C. H. Growth of bacteria in an iron-free medium. Arch. Biochem. 1 : 303-310. 1942.

19. WARING, W. S. and WERKMAN, C. H. Iron requirements of heterotrophic bacteria Arch. Biochem. 1 : 425-433. 1942.

20. WEBB, M. The influence of magnesium on cell division. I. The growth of *Clostridium welchii* in complex media deficient in magnesium. J. Gen. Microbiol. 2 : 275-287. 1948.

21. WEBB, M. The influence of magnesium on cell division. 2. The effect of magnesium on growth and cell division of various bacterial species in complex media. J. Gen. Microbiol. 3 : 410-417. 1949.

22. WEBB, M. The influence of magnesium on cell division. 3. The effect of magnesium on the growth of bacteria in simple chemically defined media. J. Gen. Microbiol. 3 : 418-424. 1949.

23. WEBER, M. M. Red halophilic micro-organisms. A problem in the biochemical foundation of an ecological specialization. Biol. Rev. City College of New York, 1 : 9-14. 1949.

SLIDE CULTURE OF TUBERCLE BACILLI

III. ROUTINE DIAGNOSTIC TRIALS[1]

By D. M. Simpson[2] and R. W. Reed[3]

Abstract

The adaptation of a modified slide culture technique to the routine diagnosis of pulmonary tuberculosis is described in detail. Results of culture of 2248 specimens of sputum by this method and by conventional techniques are compared. The results demonstrate the superiority of the slide culture technique; 92.6% of positive-smear specimens yielded positive cultures, as against 70% by routine methods. Results were almost identical with both methods in the smear-negative group and an explanation of this apparent discrepancy is advanced. 100% of the (positive) slide cultures were positive by the end of 14 days, whereas controls reached 96.5% only at the end of eight weeks.

Since Robert Koch discovered the tubercle bacillus in 1882(4) a variety of media and methods have been introduced for the primary isolation of the organism from pathologic material. One of the problems has been to devise a procedure which would permit an earlier bacteriological diagnosis of the disease and which would be of greater assistance in clinical management during its course. It is frustrating to the clinician to have to wait for three to eight weeks, or longer, for a bacteriological report on his patients, as is the case where the conventional cultural methods are used. On many occasions the first evidence of relapse is the reappearance of tubercle bacilli in the sputum, in too small numbers for microscopic detection, so that rapid cultural detection becomes of paramount importance. Implied in the over-all problem, of course, is not only speed but sensitivity to very small numbers of organisms as well. The ideal would be the cultural detection of one viable tubercle bacillus, from mixed culture, on a simple, selective direct-plating medium within 24 to 48 hr. This happy state has not yet been attained for no single method fulfills all our requirements. Indeed, it is fortunate that the tubercle bacillus is such a relatively hardy organism for otherwise, considering the punishment to which we subject it, the number of positive cultures would be considerably less than at present.

In 1953 Reed (6) reported the results of experiments with a modified slide culture technique for the cultivation of tubercle bacilli from untreated sputum specimens. Early in the course of these experiments it became evident that the method held much promise and it was decided to carry out full scale trials under actual diagnostic laboratory conditions. The present paper reports the results to date of these trials.

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Contribution from Camp Hill (D.V.A.) Hospital and the Department of Bacteriology, Dalhousie University, Halifax. Presented in part at the 4th annual meeting of the Canadian Society of Microbiologists at Kingston, Ont., June 24, 1954.

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Materials and Methods

Specimens

No conscious selection of specimens was made. At first, owing to certain limitations, only a representative number of specimens were taken at random from those submitted to the laboratories at Camp Hill Hospital. Soon, however, with the development of the apparatus described below, all specimens were cultured as received, both by slide culture, and by the method commonly used in this laboratory for some years. All specimens were collected in sterile wide-mouth, straight-side, medium tall, 4-oz. screw capped bottles. The average specimen measured 25 cc. and usually represented a 24 hr. collection although if sputum was scanty, the collecting period was extended to 48 hr. and rarely to 72 hr. The greatest number of specimens was received from out-patients reporting periodically for review.

Medium

(*a*) Kirchner's (3) medium was prepared as follows:

$Na_2HPO_4.12H_2O$	3 gm.
KH_2PO_4	4 gm.
$MgSO_4.7H_2O$	0.6 gm.
Sodium citrate	2.5 gm.
Asparagin (DIFCO)	5 gm.
Glycerine A.R.	20 ml.
Distilled water	1000 ml.

The final pH was adjusted to 7.2 and the medium dispensed in 20-ml. amounts into the special culture tubes (Fig. 1, *A*) described by Lowry and Berry (5). These tubes were then autoclaved for 20 min. at 121° C. and stored in the refrigerator.

(*b*) Fresh pooled human serum was received from the Public Health Laboratories of the Nova Scotia Department of Health. This serum was filtered immediately through a Seitz EK filter pad, checked for sterility, and stored in the refrigerator.

(*c*) Immediately before use 2 ml. of sterile serum (10%) was added to each of the culture tubes required.

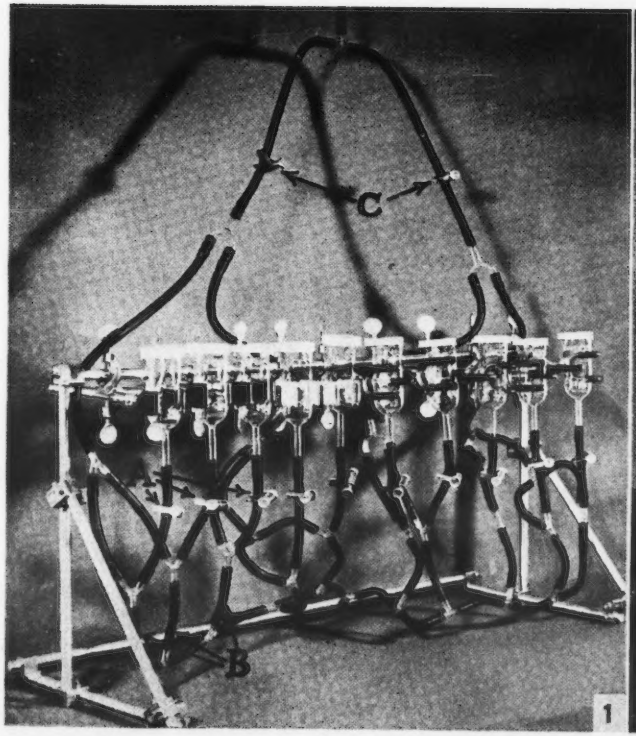
Procedure

(*a*) The sputum specimen in its container was fastened in a laboratory model paint conditioner* and agitated until completely homogenized. Shaking time varied with the specimen but was usually less than five minutes.

(*b*) From each specimen two thick smears were made on standard microscope slides previously marked by a diamond pencil with the culture number. These smears were then dried on trays in a small bench size incubator for one hour at 37° C.

(*c*) Following drying, slides were placed in the sterile decontamination apparatus pictured in Fig. 1. Only the two slides from a single specimen

* *Red Devil Tool Company, Irvington, N.J.*



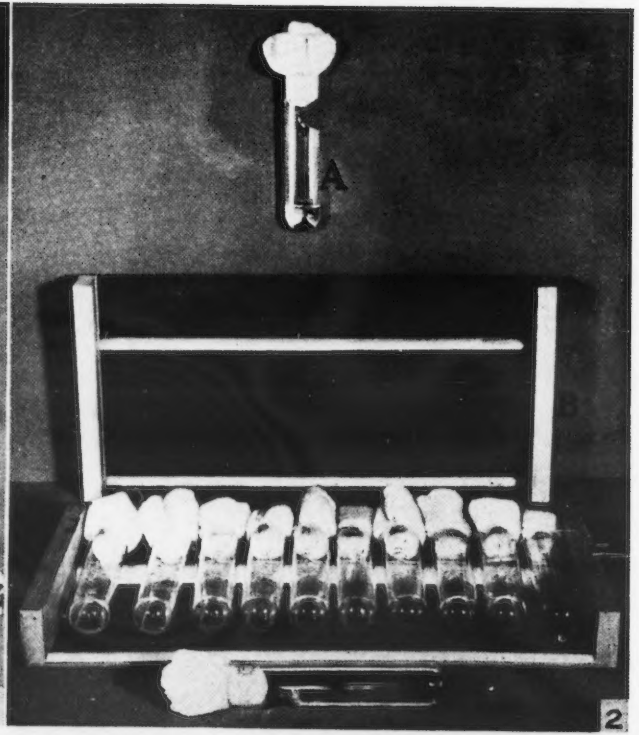


FIG. 1. 'Decontamination' apparatus. See text for explanation. FIG. 2. Slide culture tubes and incubation racks. See text for explanation.

were placed in each decontamination tube. This was to prevent false positive results since it had been shown experimentally that tubercle bacilli could pass from a positive to a negative slide when the two were placed in the same decontamination tube. Each tube was filled with a solution of 6% (v/v) sulphuric acid C.P. from a 3 liter aspirator bottle with rubber tubing outlet and hooded pipette. The acid was allowed to completely cover the slides for seven minutes at which time the pinchcocks (Fig. 1, *A*) were removed, the acid allowed to flow out and the pinchcocks replaced at the position shown in Fig. 1, *B*. The pinchcocks shown at *C* (Fig. 1) were then opened allowing sterile distilled water to flow in to all decontamination tubes simultaneously. The chambers were drained immediately by removing the pinchcocks from *B* (Fig. 1); the flushing process was repeated twice. It was shown experimentally that all traces of acid were removed by this triple washing. All discarded fluid was allowed to flow into a 12 liter bottle, containing lysol, under the bench (which was drilled to allow passage of the rubber tubing).

(*d*) Each slide was now transferred aseptically to a culture tube (Fig. 2, *A*) containing basal medium plus serum and the tubes laid horizontally on the specially constructed racks pictured in Fig. 2, *B*. The slides were placed in the culture tubes with the specimen side upward to allow maximum aeration. The amount of medium contained in the tube was sufficient to keep slides just immersed during the period of incubation. Each rack conveniently held 10 culture tubes (Fig. 2, *C*) which were prevented from rolling by the half-elliptical bar lengthwise down the rack. The tubes were marked, using a grease pencil, with the number corresponding to the slide. The racks were constructed with enough clearance on the side-pieces that they could be stacked in layers.

(*e*) One slide from each specimen was removed after six days', the other after 14 days' incubation at 37° C. All slides were dried and fixed at 90° C. for 30 min. on an electric staining bath, stained, and examined.

Staining

Staining was done cold according to the method of Aubert (1) that is, employing "Tween 80" added to the filtered fuchsin stain. However, we have customarily used pararosanilin chloride (National Aniline Co.) in a concentration of 0.3% as recommended by Yegian and Budd (7), rather than the more commonly used basic fuchsin, and have found it to give more consistently uniform results. Decolorization was in 5% nitric acid, followed by 95% ethyl alcohol, and the counterstain employed was 0.1% aqueous brilliant green, made slightly alkaline by the addition of 0.25% of 4% sodium hydroxide.

Controls

After homogenization and preparation of smears for slide-culture the specimens were digested with an equal volume of 20% trisodium phosphate for approximately 20 hr. at 37° C., then neutralized with 10% hydrochloric acid in the presence of bromthymol blue, and transferred to sterile 50-ml.

(Rockefeller Centre) centrifuge tubes. These tubes were then centrifuged at top speed (in an International Type SB size 1 centrifuge with conical head) for 30 min., the liquid decanted, and the sediments planted to tubes of Herrold's egg yolk agar by means of a 5 mm. heavy platinum loop. The inoculum was well rubbed over the whole surface of the slope. Smears were made at the same time for microscopic examination of the sediment. All cultures were examined weekly and the appearance of first growth recorded.

Decontamination Apparatus

The apparatus pictured in Fig. 1 was made up from Flexaframe parts. The whole assembly could be placed in the autoclave when the inlet tube was disconnected from the sterile water supply and outlet tube drawn up from the waste bottle. The decontamination tubes were covered with sputum bottle caps which remained in position at all times except when the acid solution was introduced. Two sets of this apparatus were made so that in two consecutive runs, 20 specimens could be handled. After use the apparatus was disconnected, the inlet and outlet tubes capped with aluminum foil, and the whole autoclaved for 30 min. at 18 lb. pressure.

Results

A comparison of results obtained with 2248 specimens examined during the period being reported is shown in Table I. Of these, 230 gave a positive smear on examination of concentrated sediment. Of this number, 150 were positive on culture by both methods, a total of 65%; 63 or 27.4% were positive by slide culture only, whereas only 11 or 4.8% were positive by routine culture only. Combination of these figures gives a total of 213 or 92.6% positive by slide culture, as opposed to 161 or 70.0% by routine culture. Only six (2.6%) specimens with a positive smear failed to yield a positive culture by one method or the other. 2018 specimens gave negative smears and of these 32 yielded positive cultures by both methods; 24 specimens were positive by slide culture only; and 25 were positive by routine only. Thus 2.7% of the negative smear specimens were positive by slide culture, and 2.8% were positive by routine culture.

TABLE I

COMPARISON OF SLIDE AND ROUTINE CULTURES OF ALL SPUTUM SPECIMENS

Conc. smear	Slide	Routine	Number	%	Slide total %	Routine total %
230 Positive	+	+	150	65.2	92.6	70.0
	+	−	63	27.4		
	−	+	11	4.8		
	−	−	6	2.6		
2018 Negative	+	+	32	1.6	2.7	2.8
	+	−	24	1.1		
	−	+	25	1.2		
	−	−	1937	96.1		

TABLE II

COMPARISON OF RESULTS OF POSITIVE SPUTUM CULTURES

Culture pos. by	Smear pos. (224)	Smear neg. (81)	Total (305)
Slide and routine	150 (67%)	32 (39.2%)	182 (60%)
Slide only	63 (28.1%)	24 (30.0%)	87 (28.2%)
Routine only	11 (4.9%)	25 (30.8%)	36 (11.8%)
Slide total	213 (95.1%)	56 (69.2%)	269 (88.2%)
Routine total	161 (72%)	57 (70.0%)	218 (71.8%)

A comparison of results of positive cultures alone is given in Table II. Of the positive smear specimens, 150 (67%) were positive by both methods; 63 (28.1%) were positive by slide culture only, in contrast to 11 (4.9%) positive by routine culture only, giving a total of 213 (95.1%) positive by slide culture and 161 (72%) positive by routine culture, a difference of 23.1% in favor of the slide method. Of those specimens having a negative smear, the total number positive by slide culture (56 or 69.2%) was almost identical with the total number positive by routine culture (57 or 70%). Considering all positive culture results it is seen that 269 (88.2%) were positive by slide culture whereas routine culture yielded 218 (71.8%) positives.

Table III gives an analysis of the positive culture results, in terms of time required to become positive, expressed as a cumulative percentage. Of the total of 269 slide cultures, 52% were positive within one week (actually six days), and 100% were positive in 14 days. Of the 218 positive routine cultures 13.8% were positive by the third week of incubation and only at the end of eight weeks did the total number approach the 100% mark.

TABLE III

COMPARISON OF TIME REQUIRED TO BECOME POSITIVE

Culture technique and No. of cultures	Incubation time, wk.											
	1	2	3	4	5	6	7	8	9	10	11	12
	Cumulative percentage positive											
Slide, 269	52	100										
Routine, 218	0	0	13.8	39	57	67.5	75.5	96.5	97	97	97	100

Discussion

The adaptation of the slide culture technique, as described, to the conditions and requirements of the diagnostic laboratory has been fully justified in several respects. The initial outlay for equipment is not great, the technique is easy to learn, large numbers of specimens can be handled with relative ease, and the results are encouraging, to say the least. Not the least important consideration from the medical standpoint is the time element involved,

making it possible for the physician to correlate clinical, radiological, and bacteriological data together, with no great lag, and, therefore, to be able to arrive at a much more satisfactory assessment of the patient's progress at the time when his status is being reviewed.

As shown in Table I and Table II the sensitivity of the slide culture method (with respect to number of positive recoveries) is considerably greater, in the smear-positive category, than the routine method; in the smear-negative group the two methods would appear to be about equal in sensitivity. However, this somewhat deceptive discrepancy between the smear-positive and smear-negative group can, we believe, be attributed solely to the very great difference in size of inoculum existing between the two methods. This discrepancy becomes particularly evident when the organisms are present in only minimal numbers as is the case in the smear-negative group. In the slide culture method the inoculum consists of a loopful of untreated, non-concentrated (although homogenized) sputum. In the routine method the inoculum is derived from the sediment obtained by concentrating the balance of the specimen, which obviously contributes a greater number of organisms to the inoculum regardless of the number present in the original specimen. Not taken into account, of course, in either method, is the deleterious effect of the "decontaminating" procedures, exposure to trisodium phosphate on the one hand and to sulphuric acid on the other. One obvious need then is for the development of a suitable concentration procedure for use with the slide culture method, and another the development of a "decontaminating" procedure which would allow all viable tubercle bacilli to survive while at the same time suppressing other organisms completely. Experiments designed along these lines are already in progress.

It is a known precept that the use of a wide variety of media will produce an over-all increase in the number of positive recoveries. Since the use of only one conventional medium as a control in these experiments might be considered as insufficient, we have had underway for some months comparative trials employing slide culture, four standard egg media, a blood medium, and guinea pig inoculation. Results of these trials will be reported at a future date; indications at present are that the slide culture method compares more than favorably.

Another problem which remains to be solved is that of distinguishing, in slide culture, between tubercle bacilli and saprophytic acid-fast organisms, and between virulent and non-virulent strains of tubercle bacilli. The latter problem is not confined to the use of the slide culture method alone. The appearance of isonicotinic acid hydrazide-resistant strains, non-virulent or with markedly attenuated virulence, for guinea pigs, poses a problem in many instances for those interested in the bacteriology of tuberculosis.

In 1954, Dubos (2) stated, "The primitive bacteriological techniques worked out decades ago for the detection of tubercle bacilli in sputum and other pathological materials are still in use today.....it is also beyond doubt that the empiricism symbolized by the use of egg and serum

media constitutes only a crude approximation to the conditions which are optimal for bacillary growth". It is hoped that the ultimate development and modification of the slide culture technique may provide at least a part of the answer to the problem of improving diagnostic methods.

Acknowledgments

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References

1. AUBERT, E. "Cold" stain for acid-fast bacteria. Can. J. Public Health, 41 : 31-32. 1950.
2. DUBOS, R. J. Unsolved problems in tuberculosis. Am. Rev. Tuberc. 70 : 391-401. 1954.
3. KIRCHNER, O. Die Leistungsfahigkeit der Tiefenkultur des Tuberkelbazillus bei Verwendung besonders geeingneter flussiger Nahrboden. Zentr. Bakteriol. 124 : 403-412. 1932.
4. KOCH, R. Uber die Aetiologie der Tuberkulose. Berlin. klin. Wochschr. 19 : 221-230. 1882.
5. LOWRY, H. and BERRY, J. W. Further observations on the technic of slide culture of the tubercle bacillus. Am. J. Clin. Pathol. 20 : 273-277. 1950.
6. REED, R. W. Slide culture of tubercle bacilli. I. Media and methods. Can. J. Med. Sci. 31 : 367-376. 1953.
7. YEGIAN, D. and BUDD, V. Ziehl-Neelsen Technique. Am. Rev. Tuberc. 48 : 1-4. 1943.

THE PHOSPHORUS FRACTIONS OF *BACILLUS CEREUS* AND *BACILLUS MEGATERIUM*

I. A COMPARISON OF SPORES AND VEGETATIVE CELLS[1]

By P. C. Fitz-James[2]

Abstract

The phosphorus fractions of the spores and young vegetative cells of *B. cereus* and *B. megaterium* were compared using the methods of Schneider and of Schmidt and Thannhauser. Differences were found, not only between the two types of cell, but also between the species studied. The spores of *B. cereus* and *B. megaterium* contained, on a dry weight basis, similar amounts of nucleic acid, yet the spores of *B. megaterium* contained twice as much total phosphorus as those of *B. cereus*. The excess phosphorus of *B. megaterium* spores was present as an acid and alkali insoluble residue and was made up of empty spore coats. While this same fraction accounted for only 4% of the total phosphorus of *B. cereus* spores it made up some 60% of that of *B. megaterium*. A similar fraction from vegetative cells was low in phosphorus. The spore coats of *B. megaterium*, in contrast to the cell walls were lysozyme-resistant. Cold acid-soluble and lipid phosphorus could be properly estimated only on disrupted spores. Disruption also proved essential for the ready extraction of spore nucleic acids with hot TCA, but did not greatly alter the solubility of the residue phosphorus. Ribosenucleic acid (RNA) comprised 50% of the total phosphorus in the spores of *B. cereus* (3–4% of the dry weight) but only 25% in *B. megaterium* spores. In both species the RNA of the vegetative forms accounted for a larger proportion of the total P of the cell. Ribonuclease digested the RNA of spores and vegetative cells to the same degree. The Schmidt and Thannhauser method was found more suitable than the Schneider method for the estimation of desoxyribosenucleic acid (DNA). The DNA content of *B. cereus* spores was about 1% of their dry weight; that of *B. megaterium* spores was slightly less. Some 12–20% of the phosphorus of *B. cereus* spores and 6% of that of the young vegetative cells was present as acid-labile (non-nucleic acid) phosphorus which exhibited some of the characteristics of polymetaphosphate.

Introduction

The changes which occur when a resting spore germinates and, in a matter of some sixty minutes, develops into a vegetative cell are presumably accompanied by marked synthetic activity. Because of the ease with which germination can be initiated, the resting spore is an ideal point from which to embark upon a study of cell growth. The main part of this work, described in the second paper of this series, deals with changes in amount of the different phosphorus fractions in synchronously germinating spores. As a basis for that study, however, the present paper compares the phosphorus fractions of resting spores with those of fully developed vegetative cells of *Bacillus cereus* and *Bacillus megaterium* by applying the methods of Schneider (31) and of Schmidt and Thannhauser (30). When the present study was begun, although various chemical and cytochemical analyses had been made of vegetative bacteria at different stages of growth (32, 16, 3), no such direct comparison existed.

[1] *Manuscript received March 4, 1955.*
Contribution from the Department of Bacteriology and Immunology and the Department of Biochemistry, University of Western Ontario, London, Ontario.

[2] *Medical Research Fellow, National Research Council of Canada.*

Materials and Methods

Stock cultures of *B. cereus* and *B. megaterium* (originally from the collection of Dr. C. F. Robinow) were maintained on potato extract agar. To secure bulk lots of spores, saline or water suspensions of these cultures were spray-inoculated into 10 to 20 Povitsky bottles, each containing 500 ml. of the agar medium of Howie and Cruikshank (12) supplemented with 0.5% casamino acids (Difco, technical). Sporulation was complete in four days. After seven days, the culture was scraped off the agar surface and collected with sterile water. The harvest was then filtered through eight layers of cheesecloth on a Buchner funnel to remove agar flakes and washed at least eight times on the centrifuge with distilled water. After each of these washings, the top of the pellet was scraped to remove lighter material (vegetative debris). The washed spores were maintained as a thick water suspension from which samples for dry weight, cell count, or phosphorus study could be pipetted with reasonable accuracy. The spores in such washed suspensions, when examined in air-mounted nigrosin films, were found to be completely refractile and apparently free of vegetative remnants.

The vegetative cells were grown in 2-litre flasks each containing 500 ml. of heart infusion broth (Difco) inoculated with a small amount of spore suspension. After the cultures were rocked vigorously in a 37° C. bath for some two to nine hours, they were quickly cooled and washed with ice-cold 0.9% (w/v) sodium chloride or 0.01 *N* sodium bicarbonate on a cold high-speed centrifuge. A heavy suspension was prepared and samples fractionated immediately.

In final suspensions the number of spores or vegetative cells was counted in a Petroff-Hauser chamber. Dilutions were made in 0.9% (w/v) NaCl containing 1% (w/v) formaldehyde. Staining procedures and photographic techniques have been described previously (9).

Dry weights were determined on duplicate samples (1–2 ml.) of the final cell suspensions pipetted to tared weighing bottles, dried by heating in a 70-90° C. oven overnight, then stored to a constant weight over phosphorus pentoxide or sulphuric acid. The spores usually survived such drying but vegetative cells did not. The phosphorus values are expressed as mgm. P/100 gm. of these dry weights.

Disruption, found to be essential for the proper fractionation of spores, was accomplished by using a Mickle (17) tissue disintegrator and micro glass beads. Four to eight milliliters of a thick cell suspension (10–25 mgm./ml. dry weight) was vibrated in a tightly stoppered glass cup with 3–4 gm. of Ballotini glass beads (size #14 for the spores and size #10 or #12 for vegetative forms). Cells were vibrated either in cold 10% (w/v) trichloroacetic acid (TCA) or in ethanol (50% to absolute) and frothing prevented by addition of 'Anti-Foam A' (Dow Corning) or tri-N-butyl citrate. The degree of disruption was followed by microscopic examination of air-mounted nigrosin films. Usually 15–30 min. of full vibration was sufficient to disrupt all cells. The suspension of disrupted cells was divided into four equal samples, providing duplicates for each method of fractionation outlined below.

Analytical Methods

The fractionation procedures of Schneider (31) and of Schmidt and Thannhauser (30) were applied to duplicate samples after the method outlined by Logan, Mannell, and Rossiter (15). In one study, the perchloric acid fractionation method (19) for separating ribosenucleic acid (RNA) and desoxyribosenucleic acid (DNA) was compared with the other methods of fractionation (using whole resting spores).

However, some modifications and additions were made. The hot (90° C.) TCA extraction of Schneider (31) had to be repeated two or three times for complete removal of nucleic acids from whole spore preparations. The extracts were then examined for total P, nucleic acid P (by both color and ultraviolet absorption tests), and occasionally for labile P. The TCA-insoluble residue was examined for total and labile P.

The Schmidt and Thannhauser (30) fractionation was modified to include the extraction of the DNA from the acidified residue (after KOH hydrolysis) with 5% (w/v) TCA for 15 min. at 90° C. as described by Schneider (31). After the estimation of DNA P by ultraviolet absorption, a portion of the extract was ashed for total P and another taken for the diphenylamine reaction (4). The residue remaining after the extraction of the DNA (the "S&T residue") was ashed for total P. The supernatant from the precipitation of DNA (the "S&T supernatant") was analyzed for total P, inorganic P, and labile P. An outline of these fractions, with the names used to describe them in this paper, is shown in Table I.

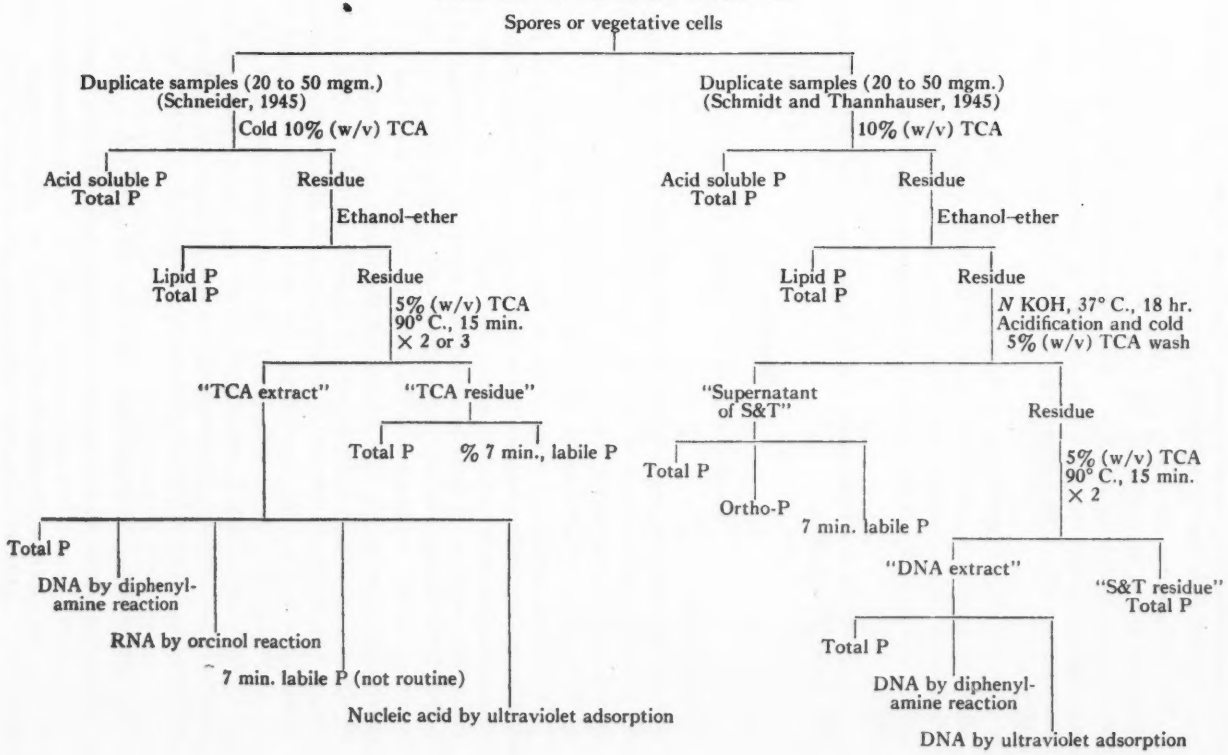
Total P was estimated on aliquots containing 5–40 μgm. P by the method of King (14) except that "metol" (*p*-methylamino-phenol sulphate) was used instead of King's reducing reagent (11). The color was read in a Coleman Universal Spectrophotometer at 650mμ.

Inorganic P (ortho-P) was determined by the modification of the method of Berenblum and Chain (1) described by Ernster, Zetterström, and Lindberg (8). Acid-labile P was studied in both residues and extracts. One portion of the residue was hydrolyzed in a glass stoppered tube with 1 *N* hydrochloric acid (final concentration) for seven minutes on a boiling water bath and another portion hydrolyzed for 14 min. After hydrolysis the cooled samples were centrifuged and the total P and ortho-P content of the supernatants estimated. The ortho-P liberated during the hydrolysis of a residue was expressed as a percentage of the total P extracted by the *N* HCl during the same time period. Extracts were likewise studied for ortho-P liberated by seven and 14 min. hydrolysis but in addition the ortho-P originally present was estimated.

In addition to the usual inorganic P standard, two other standards were essential for the accurate estimation of labile P. The first of these was the synthetic potassium polymetaphosphate ("K") preparation of Ingelman and Malmgren (13). A fresh, saturated solution of this material contained from 50–55 μgm. P/ml. Less than 1% of this was orthophosphate but from 92–94% was converted to ortho-P in seven minutes (7 min. labile P) by

TABLE I

OUTLINE OF FRACTIONATION PROCEDURES



HCl at 100° C. In addition, by estimating the rate of hydrolysis of a standard RNA, one could, if necessary, correct the 7 min. labile P of the unknown for any ortho-P liberated by hydrolysis of RNA (34).

The orcinol test for ribose was done on samples of the extract (usually diluted 1/10) as described by Schneider (31). Heating was for 30 min. and readings were made at 655 mμ. The diphenylamine reaction of Dische (4) was applied using the 37° C. incubation recommended by Patterson and Dackerman (21) rather than the 10 min. heating. Color was read at 600 mμ.

The ultraviolet estimation of nucleic acids was applied to TCA extracts after the method described by Logan *et al.* (15). The wave length recommended by these authors (268.6 mμ) was used for calculating the nucleic acid P, but in addition the character of the spectra was studied from 258–290 mμ.

Results of all determinations are presented in terms of the P content of the standard solutions.

Results

Fractionation of Whole Cells

Bacillus cereus

Although later studies revealed the necessity of disrupting resting spores for complete fractionation of their P, much information was gathered early in this study by comparing whole cells. A typical comparison of the fractions of whole spores and vegetative cells is shown in Table II.

With whole spores the temperature of the cold TCA extractions was found to affect many of the subsequent phosphorus values. The spores of Table II, column I, were sedimented from the cold acid extract in a non-refrigerated centrifuge, while with those of column II, the temperature was maintained below 2° C. throughout the extractions. As a consequence of the slight warming during centrifugation, the spores of column I were non-viable after extraction and some 25–30% underwent the structural change characteristic of acid hydrolysis (27) and associated with the removal of bound phosphorus (10). The spores of column II remained viable and refractile throughout both this and the lipid extractions. In fact, resting spores could survive for as long as 20 days in 10% (w/v) TCA at a temperature of 2° C. The acid-soluble and lipid P removed from the surviving spores (column II, Table II) can probably be considered as external in origin. The extraction of these two fractions from vegetative cells presented no such difficulty.

The Extraction of Spores and Vegetative Cells with Hot TCA

When the ultraviolet absorption test was applied to TCA extracts (method of Schneider (31)) of whole spores killed by cold TCA, the values for nucleotide P were fairly consistent with those obtained by other methods (Table II, column I). However, with spores which had survived the cold TCA and lipid

TABLE II

THE APPLICATION OF THE SCHNEIDER (31) (*A*) AND THE SCHMIDT AND THANNHAUSER (30) (*B*) PHOSPHORUS FRACTIONATION PROCEDURES TO WHOLE SPORES AND VEGETATIVE CELLS OF *B. cereus*

mgm. P/100 gm. dry weight

	Whole resting spores (water washed) I	Whole resting spores (water washed) II	Whole vegetative cells grown from spores 3-hr. culture	Whole vegetative cells grown from spores 4-hr. culture
(*A*) and (*B*) Acid-soluble P (total)	80	20	150	185
Lipid P (total)	22	17	71	100
(*A*) TCA extract (5% w/v TCA 90° C.)				
Total P	552	618	1106	1230
u-v absorption as P*	563	1105†	1031	1150
RNA P (orcinol)	470	540	910	—
DNA P (diphenylamine)	109	96	128	162
7 min-labile P (including ortho-P)	36	—	99	—
RNA P/DNA P	4.3	5.6	7.0	—
TCA residue				
Total P	157	148	56	45
Labile P	72	82	4	3
(*B*) "Supernatant of S&T"				
(1) Total P	578	625	1015	1170
(2) Ortho-P	24	41	14	9
(3) 7 min-labile P (including ortho-P)	92	85	100	105
RNA P (by difference) (1) − (3)	486	540	915	1065
"DNA extract"				
DNA P by total P	99	96	117	101
DNA P by u-v P*	110	95	119	103
DNA P by diphenylamine	95	96	120	105
RNA P/DNA P	4.7	5.6	8.5	10.5
"S&T Residue" P	48	44	26	44
Sum total P (as % dry weight)	0.819	0.803	1.380	1.580

* *Ultraviolet absorption at 268 mμ expressed as nucleic acid P.*

† *Abnormally high owing to the presence of a non-nucleotide absorbing substance. The extract showed the spectrum of dipicolinate (Powell (23)).*

extractions, these values were some one and a half to two times higher (Table II, column II). This interference was found to be associated with an ultraviolet absorption spectrum with a maximum at 271 mμ and two subsidiary peaks at 262 and 277 mμ which completely masked the absorption peak of the purines and pyrimidines of the nucleotides. The estimation of nucleic acid P was thus erroneously high. The material responsible was not associated with spore phosphorus and could be completely extracted by heating spores for 30 min. in boiling water or by any acid treatment which rendered spores non-viable. A similar substance encountered by Powell and Strange (24) in *B. megaterium* spores has been identified by Powell (23) as calcium dipicolinate. Young vegetative cells were devoid of this interfering substance (Tables II and V).

As one might suspect, the nucleic acids of whole spores were more difficult to extract than those of vegetative cells. As shown in Table IIIA, the first

TABLE III

THE REMOVAL OF PHOSPHORUS FROM SPORES AND VEGETATIVE CELLS WITH REPEATED 5% TCA (90° C.) EXTRACTIONS

	Spores		9-hr. vegetative cells	
A. The percentage distribution of extracted P *(total* P *King-Gomori)*				
1st extraction	68.3		96.8	
2nd extraction	23.4		3.2	
3rd extraction	8.3		—	
B. The percentage extracted phosphorus present as nucleic acid P				
Calculation	*a*	*b*	*a*	*b*
1st extraction	84	—†	100	94
2nd extraction	94	52	—	48
3rd extraction	29	19	—	—

NOTE: Calculation $(a) = \frac{\text{Total nucleic acid P (by color tests)}}{\text{Total P of extract}} \times 100$

$(b) = \frac{\text{Ultraviolet absorption as nucleic acid P}}{\text{Total P of extract}} \times 100.$

† *See footnote, Table II.*

extraction removed nearly all of the TCA-soluble P of the vegetative cells, but only two-thirds that of the whole spores. The interfering substance (dipicolinate) of spores was entirely removed by the first TCA extraction. From Table III*B*, it is evident that at least two extractions were necessary for complete removal of nucleic acids of the spores. In both cell types, phosphorus material other than nucleic acids was removed, particularly by the later extractions. This was usually more marked in the spores, but also occurred in young vegetative cells. As demonstrated below (see also Table II) most of this other P material was acid-labile.

TCA Residual Phosphorus

After repeated TCA extractions of whole *B. cereus* spores surprisingly large amounts of residual P remained and some 60 to 80% of it was seven minute acid-labile. The manner in which *N* HCl at 100° C. removed P from this residue and the percentage of this extracted P which was simultaneously converted to ortho-P (acid-labile) as indicated in Fig. 1. The small TCA-

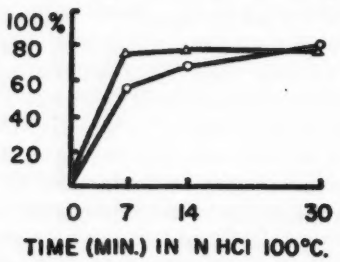


FIG. 1. Hydrolysis of TCA residue of *B. cereus* spores in 1 *N* HCl at 100° C. O — O Extraction curve of total P by the HCl. Δ — Δ % of extracted P present as ortho-P.

residue P of vegetative cells, on the other hand, was quite insoluble and non-labile towards acid. The labile part of the TCA residual P of spores was readily extracted by *N* KOH.

Comparison of the Perchloric Acid and the TCA Extraction Procedures

The two nucleic acids of spores, RNA and DNA, could be separated by the $HClO_4$ procedure of Ogur and Rosen (19). A comparison of their method with the TCA procedure is shown in Table IV. The 40 hour extraction in *N* $HClO_4$ at 4° C. killed the spores, removed the RNA P as well as the dipicolinate and produced the structural rearrangement of the spore found after other methods (27) of acid hydrolysis. The $HClO_4$-insoluble and the TCA-insoluble P were comparable in amount.

TABLE IV

COMPARISON OF THE OGUR AND ROSEN (19) (O-R) PERCHLORIC ACID AND SCHNEIDER (31) (SCHN.) TCA EXTRACTION OF NUCLEIC ACIDS FROM WHOLE SPORES (*B. cereus*)
mgm. P/100 gm. dry weight of spores

	Method of Ogur and Rosen	Method of Schneider
	0.2 *N* $HClO_4$ 0 – 2° C.	10% (w/v) TCA 0 – 2° C.
Acid-soluble P	4	10
Lipid P	4	8
Nucleic acid extracts	*N* $HClO_4$ 40 hr. 4° C.	5% (w/v) TCA 90° C., 15 min. (Twice)
Total P	363	476
u-v P*	1150†	1020†
RNA P (orcinol)	380	410
DNA P (diphenylamine)	0	82
	0.5 *N* $HClO_4$ 70° C., 15 min. (Twice)	
Total P	132	
u-v P*	96	
RNA P (orcinol)	0	
DNA P (diphenylamine)	93	
Residue P		
Alkali-soluble P (O-R)	100	
Alkali-insoluble P (O-R)	40	
TCA-residue P (Schn.)		150

*, † } *See footnotes, Table II.*

The Schmidt and Thannhauser (30) Fractionation of Spores and Vegetative Cells

This method, when applied as outlined, achieved adequate fractionation of the P of both cell types. Separate experiments not reported in detail showed that the major part of the DNA remained in the cells after the 18–20 hr. hydrolysis in *N* KOH and could subsequently be extracted by hot 5% TCA. As shown in Table II, the three different determinations in the "DNA extract" agreed reasonably well.

The labile P of both spores and vegetative cells was encountered in the supernatant fraction of Schmidt and Thannhauser, along with the RNA P and the so-called phosphoprotein P (ortho-P). A calculated value for RNA P was found by subtracting the labile P (including ortho-P) from the total in this supernatant. Estimates of RNA P by this method agreed with those of the Schneider (31) method.

On comparing the two methods of fractionation one observes that the values for DNA P obtained by applying the Dische (4) reaction to the Schneider (31) TCA extracts were somewhat higher than those obtained by the Schmidt and Thannhauser (30) procedure. Moreover, the TCA samples with the higher DNA values showed a greenish color instead of the usual blue of the Dische reaction. A proper value for DNA P in TCA extracts (Schneider method) could be obtained by adopting the suggestion made by Racker (26) of calculating the DNA from the difference in optical densities taken at two different wave lengths.

TABLE V

WEIGHT AND DNA P CONTENT OF *B. cereus* SPORES AND VEGETATIVE CELLS

	Batch No.	Average weight/cell 10^{-6} μgm.	Average DNA P/cell 10^{-10} μgm.
Spores	14	1.03	9.1
Spores	15	0.99	9.4
Vegetative cells (4 hr. from spores)	28	1.62	16.4

The average DNA P content per cell for two batches of spores and one batch of young vegetative cells is shown in Table V. The average concentration of DNA P per spore (9.2×10^{-10} μgm.) was comparable to that present in other microorganisms, for example, yeasts (62×10^{-10} μgm./cell (18)). The four-hour cells of Table V are illustrated in the paper following (Fig. 9, four hours, sub 60 min.). Although cells often remained attached after division, the cell count was made on the number of walled-off units. During this period of growth, the average DNA P per cell had increased 1.8-fold over the spore level. The nuclear material in photomicrographs of over a hundred of these four-hour cells (Fig. 9, following paper) was compared in size with that present in resting or early-germinating spores (before nuclear growth). The increase in nuclear structures (1.75) closely resembled the increase in DNA P during the four hours of vegetative growth. This suggests that the DNA content per cell is an expression of the amount of nuclear material present and that the DNA content of the basic nuclear structure of both spores and vegetative cells is the same.

Bacillus megaterium

Like *B. cereus*, this organism showed differences in the P distribution of its two cell types (Table VI). The abnormally high "u-v P" (ultraviolet P)

values of the spores again indicate the presence of dipicolinate. In the TCA extracts (method of Schneider (31)) of the vegetative cells, some unknown chromogen produced DNA P values much higher than those obtained by the Schmidt and Thannhauser procedure. Yet with the spores of this species, DNA P by both methods showed reasonable agreement.

TABLE VI

PHOSPHORUS FRACTIONS OF SPORES AND VEGETATIVE CELLS. APPLICATION OF SCHNEIDER (*A*) AND SCHMIDT–THANNHAUSER (*B*) PROCEDURES TO WHOLE CELLS OF *B. megaterium*
mgm. P/100 gm. dry weight

	Spores	Vegetative cells (3 hr. growth)
(*A*) and (*B*) Acid-soluble P	42	460
Lipid P	8	76
(*A*) TCA extract		
Total P	516	945
u-v P*	724†	915
Total P minus u-v P	—	30
RNA P (orcinol)	348	782
DNA P (diphenylamine)	80	145
RNA/DNA	4.35	5.4
TCA Residue		
Total P	1130	109
Labile P	110	<10
(*B*) "Supernatant of S&T"		
Total P	513	891
Ortho-P	82	9
Labile P (including ortho-P)	80	9
RNA P by difference	431	882
"DNA extract"		
DNA P by total P	88	48
DNA P by u-v P*	88	53
DNA P by diphenylamine	89	50
RNA/DNA	4.9	15.4
"S&T residue" P	1050	85
Sum total P (as % dry wt.)	1.69	1.59

*, † *See footnotes, Table II.*

The most striking characteristic of *B. megaterium* lay in the unusually high residual P of its spores. When labile P studies were applied to this TCA residue, only 8.2% of the P was extracted by the *N* HCl at 100° C. in seven minutes, and 9.8% in 14 min. Of this extracted P, only 10–11% was simultaneously converted to ortho-P by the hydrolysis. This could mean that some 10% of the total residue was acid labile. This amount is indicated in Table VI. Yet the amount of labile P in the "supernatant of S&T" was, in most instances, adequately accounted for by the ortho-P (phosphoprotein P of Schmidt and Thannhauser (30)). An occasional batch of *B. megaterium* did show small amounts of non-nucleotide labile P, but these were of doubtful significance. The high residual P of the Schneider method is reflected in the high level of the "S&T residue" P. It was always at least 20 times the amount

present in the same fraction of *B. cereus* spores. This residue was found, in both species studied, to be composed of empty spore coats, while that of vegetative cells was mostly cell walls. The high residue P of the vegetative cells shown in Table VI is due to the spore coat material left by the spores from which these three-hour vegetative cells were grown (thick germination culture). The drop in residue P from the spore level indicates the dilution of this fraction by the vegetative growth.

Lysozyme Treatment of Spore Coats and Vegetative Cell Walls of B. megaterium

In a preliminary attempt to characterize this spore P, a mixture of spore coats and vegetative cell walls (S&T residual material of germinated spores) was treated with lysozyme for 5–20 min. The cell walls were quickly and completely dissolved, leaving the empty spore coats. Only negligible amounts of P were removed by the lysozyme digestion. Thus, the spore coats of *B. megaterium*, in contradistinction to the cell walls, are rich in phosphorus and are lysozyme-resistant. An electron micrograph of a spore coat preparation obtained from 60-min. germinated spores by differential centrifugation is shown in Fig. 2*a* and that of another preparation after lysozyme digestion in Fig. 2*b*. No difference in electron density of the two preparations is noted.

Fractionation of Disrupted Spores

Following the above work, it was found that disruption of spores was necessary for the proper removal of the small amounts of lipid and acid-soluble P present. The effects of disruption on the subsequent fractionation of spore P are shown in Table VII for both *B. cereus* and *B. megaterium*. Spores disrupted in ethanol were subjected to the lipid extraction before the cold-acid extraction while those disrupted in TCA were fractionated in the normal order (Table I). It can be seen that the extraction of nucleic acids from disrupted spores by one hot TCA treatment (method of Schneider (31)) was more complete than from whole spores. This is shown by the differences between the second TCA extracts of Table VII and also in the greater amount of DNA P in the first extract. When the calculations applied in Table III to TCA extracts of whole spores were applied to those of smashed spores, contrary to the findings shown in Table III, no difference was found in the solubility of spore and vegetative cell nucleic acids. The fractionation of vegetative cell P was not altered by previous disruption in ethanol.

After disruption, the amount of P in the TCA residue of *B. cereus* spores was only slightly reduced and this fall was balanced by an increased extraction of labile P into the TCA. Total RNA P was also sometimes reduced (Expt. III, Table VII) and this reduction could be accounted for by incomplete recovery of RNA-containing material from the alcohol in which the spores were disrupted. DNA recovery after disruption was complete.

The TCA extracts (method of Schneider (31)) of disrupted spores were free of dipicolinate. Ultraviolet estimations for nucleic acid P on such extracts were now within the limits of the total P present. The dipicolinate was located in the cold acid extracts of the smashed spores.

TABLE VII

EFFECT OF DISINTEGRATION ON THE TCA EXTRACTION (SCHNEIDER (31)) OF SPORE PHOSPHORUS

mgm. P/100 gm. dry weight whole spores

	B. cereus spores						*B. megaterium* spores	
	Expt. I 10% TCA		Expt. II 50% Alcohol		Expt. III Absolute alcohol		Absolute alcohol	
	Whole	Smashed	Whole	Smashed	Whole	Smashed	Whole	Smashed
Lipid P total	1	11	0	68	0	49	8	56
Acid soluble P total	5	62	11	49	10	52	42	67
1st 5% TCA, extract (90° C.)								
Total P	525	511	513	505	518	440	474	478
u-v P*	1380†	625	1150†	530	1162†	464	704†	480
RNA P (orcinol)	450	465	460	440	485	435	348	358
DNA P (diphenylamine)	66	100	52	64	99	103	65	70
Labile P	25	48	19	56			—	—
2nd 5% TCA extract								
Total P	100	50	81	35	Pooled with first extract		42	22
u-v P*	72	23	59	12			21	11
Labile P	25	27	15	22			—	
Sum of TCA extracts								
Total P	625	561	594	540	Pooled with first extract		516	500
u-v P*	1450	648	1200	542			724	491
Labile P	50	75					—	—
TCA residue								
Total P	250	208	250	188	170	149	1130	1080
Labile P	130	106	130	100			—	—
Sum total P	881	842	855	845	698	690	1696	1690

*, † *See footnotes, Table II.*

THE NATURE OF THE LABILE PHOSPHORUS OF *B. cereus* SPORES

(a) *Resistance to Ribonuclease*

Table VIII shows a study of the effect of ribonuclease on spores disrupted‡ in absolute alcohol and on whole vegetative cells fixed in hot alcohol. The purpose of these experiments was to decide whether the acid-labile P found in the TCA residue of *B. cereus* spores was influenced by ribonuclease, and thus possibly was combined with it or related to it in structure.

Ribonuclease used in the digestion studies was the crystalline product obtained from the Worthington Biochemical Laboratory. Bulk digestion was carried out on water suspension of spores previously disrupted in ethanol and ether-dried and on similar suspensions of ethanol-fixed (10 min. in boiling alcohol) vegetative cells. Tubes containing 20–50 mgm. (dry weight) of material per milliliter of water were mixed with 4 ml. of ribonuclease solution (0.1 mgm./ml.) and incubated at 37° C. for two to four hours. Distilled water controls were run simultaneously. The progress of the ribonuclease

‡ *Whole spores resist the action of ribonuclease.*

TABLE VIII

RIBONUCLEASE DIGESTION OF DISINTEGRATED SPORES AND WHOLE VEGETATIVE CELLS OF *B. cereus*. FRACTIONATED BY BOTH SCHNEIDER (*A*) AND SCHMIDT–THANNHAUSER (*B*) PROCEDURES

mgm. P/100 gm. dry weight of whole cells

	Disintegrated spores		Whole vegetative cells (10 hr. culture)	
	2 hr. in water	2 hr. ribonuclease	4 hr. in water	4 hr. ribonuclease
Total P removed by treatments	125	400	392	1120
(*A*) and (*B*) Lipid P	15	14	60	57
Acid soluble P	40	81	25	23
(*A*) 5% TCA extracts total P (Schneider)	452	157	990	230
u-v P*	427	114	980	140
RNA P (orcinol)	330	<90	886	154
DNA P (diphenylamine)	99	95	—	—
TCA Residue P	170	177	62	69
(*B*) "Supernatant of S&T"				
(1) Total P	440	164	875	178
(2) Ortho-P	17	18	5	4
(3) Labile P, total	90	98	62	13
(4) Labile P as % of total P (1)	20%	60%	7.1%	7.3%
RNA P by difference (1) − (3)	350	66	832	160
"DNA extract"				
DNA P by total P	96	104	146	145
DNA P by diphenylamine	73	74	150	152
"S&T residue" P	60	58	27	26

* *See footnote, Table II.*

digestion was followed by staining smears of the material with gentian violet. The digested material was washed on the centrifuge with distilled water and the amounts of the P fractions remaining compared to those of undigested controls.

The large amount of P removed by the ribonuclease from disrupted spores (Table VIII) can be accounted for by a decrease in RNA. Yet some 15–20% of the total RNA remained protein-bound. The P removed by the water (control) was also largely RNA in origin. The residual or labile P of the spores did not appear to be altered by the ribonuclease. Lipid P and DNA P also were unchanged.

Ribonuclease depleted whole fixed vegetative cells of all but 13% of their RNA. A small quantity of labile P originally present in these 10-hr. vegetative cells was not encountered after digestion, indicating that it had been removed with the RNA (Table VIII).

(*b*) *Solubility Studies of the Labile P of B. cereus Spores*

Methods of estimating and extracting bound acid-labile P from other cells were applied to the spores of *B. cereus*. The direct *N* HCl hydrolysis (at 100° C.) recommended by Ebel (6) for estimating total labile P in vegetative

bacteria was applied to cold-acid and lipid-extracted spores and found to remove only 60% of the seven minute acid-labile P (corrected for nucleate P). The remainder could be readily extracted by *N* alkali.

One likely material responsible for the labile P with these solubility characteristics is polymetaphosphate. On this supposition, the mild alkali extraction method of Wiame (34) was applied to whole spores after cold TCA and lipid extraction (Group *a*) and to similarly treated spores extracted with hot TCA after the method of Schneider (31) (Group *b*). Group "*a*" was rendered non-viable by slight warming of the 10% TCA to 15° C. for 10 min. during extraction of the cold acid-soluble P. These residues were given three one-hour extractions with dilute NaOH, pH 8–9, at room temperature. The pooled alkali extract from the first study contained some 30% of the bound P. Some 47% of the P in this extract was non-nucleate labile P and 64% of this precipitated as the barium salt at pH 4.5 (carried out as described by Wiame (34)). This alkali extraction of whole, dead spores was subsequently found to have reduced by one half the TCA residual P (Schneider fractionation (31)).

When Group *b* was subjected to this mild alkali extraction, 80% of the total residual P was removed. Fifty per cent of this was acid-labile (non-nucleic acid) P and of this 96% was recoverable by barium precipitation at pH 4.5. When the washed barium precipitates of Groups *a* and *b* were mixed with two to three drops of a solution of toluidine blue (0.003%) they underwent the metachromatic change from blue to purple shown by polyphosphates. The extraction of the labile P by mild alkali was improved slightly when this TCA residue was disintegrated.

When stronger alkali extraction (*N* KOH, Schmidt and Thannhauser (30)) was applied to the TCA residue of whole spores, all but the small stable P fraction was removed. From 70–90% of the P so extracted was seven-minute acid labile (including some 10–12% ortho-P). Barium (and lead) precipitates from these extracts, although containing labile P, gave doubtful or negative metachromatic reactions with toluidine blue. This was to be expected, since it has been shown (7) that *N* alkali will degrade polyphosphate into small, non-metachromatic units.

As shown above, vegetative cells also contain labile-P, the solubility of which is more consistent with that described for the polyphosphate of other forms (29, 34, 7). Experiments designed to establish the identity of vegetative cell labile P are in progress.

The electron micrographic appearance of the TCA residue of *B. cereus* spores is shown in Fig. 3*b*. Untreated resting spores are shown for comparison (Fig. 3*a*). The nucleic acid free material (Fig. 3*b*) took up basic dyes and showed an indefinite metachromatic variation with toluidine blue. The same material after *N* KOH extraction (18 hr. 37° C.) of the labile P is shown (water washed) in Fig. 3*c*. The contracted centers of these preparations were now less basophilic and invariably orthochromatic (blue with toluidine blue).

The reversal of these two procedures, that is, alkali extraction followed by hot TCA extraction, yielded preparations composed almost entirely of empty spore-coats (an example of which appears in Fig. 3*c*).

The Effect of Aging and Alkali Washing on the P Fractions of B. cereus Spores

Throughout these studies, both acid-soluble P and residue P of *B. cereus* spores showed considerable variation. Since alkali (pH 8–9) washing of TCA-damaged spores markedly reduced their residue labile P, the question arose: could this labile P be a contaminant left by insufficient washing of the spore pellet? Moreover, heat activation (85° C. for 10 min.), the treatment used to ensure a high percentage of germination, removed as much as 50% of the residue P from some lots of water-washed *B. cereus* spores, particularly from those with a high residual P, and did not alter the other protein-bound fractions. Thus, the washing procedures were studied in relation to the P fractions.

At first, water washes were applied as outlined in "Methods", until the preparation was microscopically free of vegetative cells and the P in the wash water had fallen to less than 5 mgm. per 100 gm. of spores. It was then noticed that overnight aging of spores in water eluted from four to eight times this amount of P from the water-washed, resting spores. Moreover, mildly alkaline washes (0.004 *M* sodium hydroxide or 0.1 *M* sodium bicarbonate, pH 9–10) also removed similar amounts of P material. In a study of both aging and alkaline washing, a large suspension of water-washed spores was aged overnight in water at pH 10 and then centrifuged and rewashed twice with water. Some 70% of the P in the clear supernatant was acid-labile and precipitated by barium at pH 4.5. The washed barium precipitate showed a definite metachromatic reaction with toluidine blue. When such additional washings and agings were applied to fresh water-washed spores, the concentration of residual P was smaller and only slightly reduced by previous heat treatment. Moreover, during the overnight alkali washing, the commencement rate of germination had risen markedly and subsequent heat activation now only slightly increased it*. The effect of overnight alkali washing on the commencement of germination is similar to that shown in Fig. 1 of the following paper for spores aged four days. These preliminary studies associating an improved ability to germinate with a removal of residue (labile) P suggest that the labile P complex may be contributing to spore dormancy.

The apparent leakage of P from spores suspended overnight in water prompted some studies of the effect of prolonged storage on the P fractions of *B. cereus* spores. Preliminary results indicate a progressive and temperature-sensitive conversion of the residual or labile P into water or acid-soluble P during long periods (months) of storage. The possibility that this P is actually external and derived from vegetative remnants is realized but as yet not established. Prolonged storage (10 months) did not alter the spore content of dipicolinate.

* *The effect of aging on the ability of spores to commence germination has been observed previously (22).*

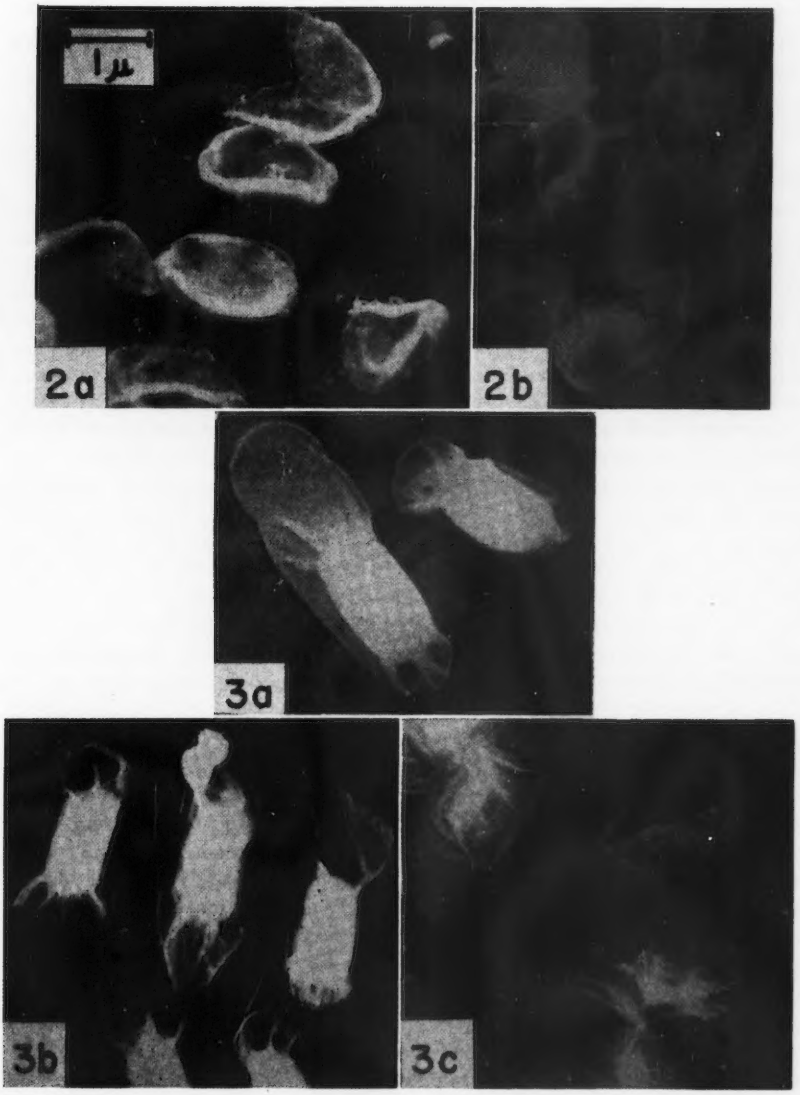


FIG. 2. Electronmicrographs of the spore coats of *Bacillus megaterium;* 2*a*, before and 2*b*, after treatment with lysozyme. FIG. 3. Electronmicrographs of the spores of *Bacillus cereus;* 3*a*, untreated resting spores; 3*b*, after extraction of nucleic acids with hot 5% (w/v) TCA (TCA residue); 3*c*, after *N* alkali extraction of the TCA residue (an empty spore case shows the intimate structural relationship of the spore coat and exosporium).

Discussion

Because of the resistant nature of bacterial spores, disruption was an essential preliminary to fractionating their phosphorus. This maneuver permitted the proper extraction of cold acid and lipid soluble substances which can interfere with the estimation of the protein-bound phosphorus fractions. Cell disruption was not essential for the fractionation of vegetative cell phosphorus.

Since the ribonucleic acid content of a cell is closely related to its growth and activity, one might expect that a completely resting and non-respiring body such as the spore, would contain little or none (3). The recent studies of Pardee (20) which indicate that the RNA of the bacterial cell is largely a by-product of synthetic activity would tend to confirm this view. On the other hand, in early ultraviolet photographs at 2750 Å (35) spores appear even more dense than vegetative cells while at 2570 Å (16) they are as dense as young vegetative forms. This high optical density of spores to ultraviolet light, particularly at 2750 Å is probably due to the high content of dipicolinic acid (23, 24), which has a peak near this wave length, as well as to the nucleic acids present. In the present study, a considerable amount of the total P of spores existed as nucleic acids. However, the amount (on a dry weight or per cell basis) was much lower in the spores than in the vegetative cells, the difference being due to the RNA content. Even so, spores contain three times as much RNA as they do DNA. The lower RNA content of spores does not necessarily mean that the *concentration* of RNA in the "cytoplasmic material" of spores is lower than that in the young vegetative cells. It probably reflects the small amount of cytoplasm present since a greater proportion of the spore mass is occupied by non-cytoplasmic materials (i.e. coats and exosporia).

Previous workers studying staphylococci (25) and *Bacterium lactis aerogenes* (2) have found the average DNA content per bacterial cell to be reasonably constant under a variety of conditions. In the present studies, the average DNA per *Bacillus* spore was constant for a number of batches of each species grown on the same medium. The average DNA content of the young vegetative cells was approximately twice that of the spores and closely related to the amount of stainable chromatin. Thus, the spore, with a single nuclear structure, contains about half the DNA of the "binucleate" vegetative cell. In the paper following, the amounts of DNA and of chromatin per cell will be examined more closely during the process of germination.

The finding here of a metaphosphate type of labile P in *B. cereus* spores and vegetative cells differs from the observations of Ebel (7), who analyzed 24-hr. cells of the anthrax variety of *B. cereus* and found no labile P which could be considered as polyphosphate. The reduction of the labile residue P by more thorough washing and by aging suggest that it may be partly or entirely external and derived from vegetative debris. Metachromatic granules (presumably metaphosphate) have been observed in *Bacillus* cells during sporulation (5) and could presumably contaminate the surface of the free

spores. The effect of more extensive washing on the labile P content is presently being studied on the spores of a number of *Bacillus* species to decide whether the labile P is an integral part or a contamination of resting spores.

It is well known that lysozyme attacks the vegetative cells of *B. megaterium* (33) and recent studies by Salton (28) have shown mechanically isolated and washed cell walls to be the substrate. In the studies here, vegetative cells of *B. megaterium* were extracted by *N* KOH, followed by hot TCA. This left a residue composed entirely of vegetative cell walls. These phosphorus-free cell walls were still readily digested by lysozyme, but the enzyme could not attack the phosphorus-containing spore coats of this species in similar residual material.

When the distribution of the phosphorus in spores is compared with that in vegetative cells, certain differences become apparent. In both species studied, RNA P accounts for a larger proportion of the total P in the vegetative cell than it does in the spore. In *B. megaterium* this difference is accentuated by the larger proportion of P found in the spore coats. The proportion of the total P present as DNA is, on the other hand, lower in vegetative cells than in spores. Outside of these nucleic acid proportions, the major difference between spore and vegetative cells was found on comparing their acid soluble, lipid, and residue P fractions. Vegetative cells contain a much larger proportion of their P in the soluble forms and a lower proportion as residual P. With these differences at hand, a study was made of the changes in the amounts of the P fractions as spores developed into vegetative cells.

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References

1. BERENBLUM, I. and CHAIN, E. An improved method for the colorimetric determination of phosphate. Biochem. J. 32 : 295–298. 1938.
2. CALDWELL, P. C. and HINSHELWOOD, C. The nucleic acid content of *Bact. lactis aerogenes*. J. Chem. Soc. Part II: 1415–1418. 1950.
3. CALDWELL, P. C., MACKOR, E. L., and HINSHELWOOD, C. The ribose nucleic acid content and cell growth of *Bact. lactis aerogenes*. J. Chem. Soc. Part IV, 3151–3155. 1950.
4. DISCHE, Z. Über einige neue charakteristische Farbreactionen der Thymonuclein sönne und eine micromethod zur Bestimmung derselben in tierischen organen mit Hilfe dieser Reaktionen. Mikrochemie, 8 : 4–32. 1930.
5. DELAPORTE, B. Observations on the cytology of bacteria. Advances in Genet. 3 : 1–32. 1950.
6. EBEL, J. P. 1st Intern. Congr. Biochem. Abstr. pp. 289, 335, and 321. 1949.
7. EBEL, J. P. Recherches sur les polyphosphates contenu dans diverses cellules vivantes. Bull. soc. chim. biol. 34 : 491–504. 1952.
8. ERNSTER, L., ZETTERSTRÖM, R., and LINDBERG, O. A method for the determination of tracer phosphate in biological material. Acta Chem. Scand. 4 : 942–947. 1950.

9. FITZ-JAMES, P. C. The structure of spores as revealed by mechanical disruption. J. Bacteriol. 66 : 312–319. 1953.
10. FITZ-JAMES, P. C., ROBINOW, C. F., and BERGOLD, G. H. Acid hydrolysis of the spores of *B. cereus*. A correlation of chemical and cytological findings. Biochim. et Biophys. Acta, 14 : 346–355. 1954.
11. GOMORI, G. A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. 27 : 955–960. 1941.
12. HOWIE, J. W. and CRUIKSHANK, J. Bacterial spores as antigens. J. Pathol. Bacteriol. 2 : 235–242. 1940.
13. INGELMAN, B. and MALMGREN, H. Investigations on metapnosphate of high molecular weight isolated from *Aspergillus niger*. Acta Chem. Scand. 4 : 478–486. 1950.
14. KING, E. J. The colorimetric determination of phosphorus. Biochem. J. 26 : 292–297. 1932.
15. LOGAN, J. E., MANNELL, W. A., and ROSSITER, R. J. Estimation of nucleic acid in tissue from the nervous system. Biochem. J. 51 : 470–482. 1952.
16. MALMGREN, B. and HEDEN, C. G. Studies of the nucleotide metabolism of bacteria. I. Ultraviolet microspectrography as an aid in the study of the nucleotide content of bacteria. Acta Pathol. Microbiol. Scand. 24 : 417–436. 1947.
17. MICKLE, H. A tissue disintegrator. J. Roy. Microscop. Soc. 68 : 10–12. 1948.
18. OGUR, M., MINCKLER, S., and MCCLARY, D. O. Desoxyribonucleic acid and the budding cycle in the yeasts. J. Bacteriol. 66 : 642–645. 1953.
19. OGUR, M. and ROSEN, G. The nucleic acids of plant tissues. I. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25 : 262–276. 1950.
20. PARDEE, A. B. Nucleic acid precursors and protein synthesis. Proc. Natl. Acad. Sci. U.S. 40 : 263–270. 1954.
21. PATTERSON, E. K. and DACKERMAN, M. E. Nucleic acid content in relation to cell size in the mature larval salivary gland of *Drosophila melanogaster*. Arch. Biochem. and Biophys. 36 : 97–113. 1952.
22. POWELL, J. F. Factors affecting the germination of thick suspensions of *B. subtilis* spores in L-alanine solution. J. Gen. Microbiol. 4 : 330–338. 1950.
23. POWELL, J. F. Isolation of dipicolinic acid from spores of *Bacillus megaterium*. Biochem. J. 54 : 210–211. 1953.
24. POWELL, J. F. and STRANGE, R. E. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. 54 : 205–209. 1953.
25. PRICE, W. H. Phage formation in *Staphylococcus muscae* cultures. XI. The synthesis of ribonucleic acid, desoxyribonucleic acid and protein in uninfected bacteria. J. Gen. Physiol. 35 : 741–751. 1951.
26. RACKER, E. Enzymatic synthesis and breakdown of desoxyribose phosphate. J. Biol. Chem. 196 : 347–365. 1952.
27. ROBINOW, C. F. Observations on the structure of spores. J. Gen. Microbiol. 5 : 439–457. 1951.
28. SALTON, M. R. J. Cell structure and the enzymatic lysis of bacteria. J. Gen. Microbiol. 9 : 512–523. 1953.
29. SCHMIDT, G., HECHT, L., and THANNHAUSER, S. J. The enzymatic formation and the accumulation of large amounts of a metaphosphate in baker's yeast under certain conditions. J. Biol. Chem. 166 : 775–776. 1946.
30. SCHMIDT, G. and THANNHAUSER, S. J. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoproteins in animal tissues. J. Biol. Chem. 161 : 83–89. 1945.
31. SCHNEIDER, W. C. Phosphorus compounds in animal tissues. J. Biol. Chem. 161 : 293–303. 1945.
32. VENDRELY, R. and LEHOULT, K. Les acides ribo– et desoxyribonucleiques de la cellule bacterienne et leur signification. Compt. rend. 222 : 1357–1359. 1946.
33. WELSHIMER, H. J. and ROBINOW, C. F. The lysis of *Bacillus megaterium* by lysozyme. J. Bacteriol. 57 : 489–499. 1949.
34. WIAME, J. M. The occurrence and physiological behaviour of two metaphosphate fractions in yeast. J. Biol. Chem. 178 : 919–929. 1949.
35. WYCKOFF, R. W. G. and TER LOUW, A. Some ultraviolet photomicrographs of *B. subtilis*. J. Exptl. Med. 54 : 449–451. 1931.

A SIMPLE METHOD OF PREPARING AN ANTIGEN FROM *HISTOPLASMA CAPSULATUM* FOR USE IN THE COMPLEMENT FIXATION TEST[1]

By N. A. Labzoffsky and J. B. Fischer

Abstract

A simple method for preparation of complement fixing antigen from *Histoplasma capsulatum* is outlined. The procedure consists of extraction of the sedimented yeast phase growth with pyridine for two hours and subsequent washing of the extracted sediment three times with buffered saline solution. The antigen so prepared is non-viable, potent, stable, and not anticomplementary; it appears to be suitable for use in routine diagnostic work.

Introduction

Results reported during the last few years by a number of workers (1, 2, 3, 4, 6, 8, 10, 11) suggest that the complement fixation test may be of considerable aid in the diagnosis of histoplasmosis. This test, however, has not as yet received wide application. This fact, perhaps, is partially due to a lack of a simple and reliable procedure for preparation of a potent and specific antigen.

Mycelial filtrate antigen (histoplasmin), besides lacking in specificity (9) and being "subject to wide variation in potency" (12), has the serious disadvantage of reacting only with sera from patients in the later stages of the illness (4). Yeast phase antigens, hitherto described, while free of the latter disadvantage (3), have not appeared to be entirely satisfactory owing to lack of both potency (2) and specificity (4).

In the present communication a simple procedure for the preparation of a potent and stable crude antigen from the yeast phase growth of *Histoplasma capsulatum* is outlined. The results obtained in this laboratory with this antigen so far indicate that it may be suitable for use in the routine laboratory diagnosis of histoplasmosis.

Material and Methods

Culture

A strain of *H. capsulatum* No. 467, originally isolated from a resident of Prescott, Ontario, was grown on Francis' cystine agar at 37° C. Seven-day-old yeast type growth was employed throughout this work.

Diluent

A 0.85% sodium chloride solution, containing 0.005 *M* phosphate buffer of pH 7.2, was used as a diluent in preparation of the antigen and unbuffered 0.85% sodium chloride solution was used in the complement fixation tests.

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Preparation of Antigen

H. capsulatum culture was collected into buffered saline solution, washed once (4200 r.p.m. for 20 min.), and the sediment extracted with 20 times its volume of pyridine*. The treatment was allowed to continue for two hours at room temperature with periodic shaking for better extraction. Next, the material was centrifuged and the sediment washed three times with buffered saline solution and resuspended in the same diluent (approximately 1 : 20 by volume). The resulting material represented the crude antigen and, after proper dilution, was employed in the complement fixation test without further treatment.

The antigen so prepared was found to be non-viable.

Sera

Sera from experimental animals and human donors were used in these experiments.

Rabbit Immune Sera

Normal rabbits about three months old were used for the production of immune serum. Procedure of immunization was the same as that described for *Cryptococcus neoformans* (5) and consisted of three subcutaneous doses of formalin killed vaccine, followed by weekly intravenous injections of viable organisms in gradually increasing doses.

Samples of blood were collected from each animal prior to each injection and the animals were bled out when the titer of the serum was reasonably high, usually five weeks after the first injection with live culture.

Human Immune Sera

Human sera, classified as immune, were obtained from the following donors:

Patient No. 1. Clinical histoplasmosis. Skin test positive. *H. capsulatum* isolated.

Patient No. 2. Clinical histoplasmosis. Brother of Patient No. 1. Skin test positive.

Patient No. 3. Clinical histoplasmosis. Skin test negative during acute stage of illness, but became skin test positive six weeks later. The first sample of blood was collected after the skin test became positive.

Patient No. 4. Clinical histoplasmosis. Skin test positive.

Miscellaneous Human Sera

This group included sera from known tuberculous patients and sera submitted for routine tests for syphilis. The latter sera were selected at random. No skin sensitivity data on the donors of these specimens were available.

All sera were kept frozen at −50° C. and for tests were inactivated at 58° C. for 30 min.

* *Pyridine, C.P., E and A tested purity reagent. Fisher Scientific Company Ltd.*

Complement Fixation Test

Technique of the test used in this work was the same as that described earlier (5). Two units of antigen were used in titration of sera.

All the usual controls were always included in each test, but for the sake of brevity were omitted from the tables in the text.

When more than one serum sample from a single patient was available, titration of all the samples was performed simultaneously.

Results

Several batches of crude antigen were prepared according to this method. Each batch was tested for antigenicity, specificity, and anticomplementary qualities.

The results of one of the titrations for antigenicity, using immune rabbit serum 1 : 40, and for anticomplementary qualities are recorded below.

Antigen 1:	8	16	32	64	128	256
Test	++++	++++	++++	++++	++++	++
Antig. control	−	−	−	−	−	−

The results recorded in this table indicate that an antigen prepared by this method is relatively potent. The potency, it was revealed, did not vary significantly from batch to batch and in no case has the antigen been found to be anticomplementary in the dilutions tested. The potency of the antigen remained unaltered during four months storage at 4° C.

Results with Patient's and Experimental Sera

Serum samples from one proved case of histoplasmosis, from three patients with clinical picture suggestive of histoplasmosis, and several samples of experimental sera were tested against this antigen and it was found that the antigen reacted equally well with human as with experimental immune sera. The results of these titrations are summarized below.

HIGHEST DILUTION OF THE SERUM GIVING COMPLETE (4 PLUS) FIXATION

Rabbit No. 554		Patient No. 2	
Oct. 15th	0	May 31st	1 : 128
Oct. 22nd	1 : 8	June 29th	1 : 128
Oct. 29th	1 : 16	Jan. 10th	1 : 128
Nov. 5th	1 : 64		
Nov. 12th	1 : 128	Patient No. 3	
		Jan. 6th	1 : 256
Patient No. 1		Jan. 14th	1 : 256
May 7th	1 : 32	Feb. 9th	1 : 128
June 22nd	1 : 64		
June 29th	1 : 64	Patient No. 4	
Aug. 3rd	1 : 64	Nov. 10th	1 : 64
Nov. 21st	1 : 128	Dec. 19th	1 : 64
		Jan. 1st	1 : 128

Results with Miscellaneous Sera

To determine the specificity of the antigen, 50 sera from tuberculous patients and 400 random sera submitted for routine Wassermann test were tested with this antigen. None of these sera, when diluted 1 : 8, reacted with

two units of the antigen. When, however, four units of the antigen were employed in the test, one of the sera from "Wassermann group" gave positive reaction and titered 1 : 32. This serum was from a woman in the last stage of pregnancy and to the knowledge of the attending physician the donor had no history suggestive of histoplasmosis. Unfortunately, the skin test was not performed on this patient.

To test further the specificity of this antigen, 100 sera from "Wassermann group" were retested, using a four-hour incubation at 4° C. This period of incubation is used in other laboratories as a standard procedure and it was reported (4) that under such conditions the yeast phase antigen yielded 5.9% to 7.7% non-specific reactions. In the present test no non-specific reactions were observed with the 100 sera tested.

In addition to the above, sera prepared against *Cryptococcus neoformans* and *Blastomyces dermatitidis* and sera from known cases of coccidioidomycosis* were also included in this test. No reaction was observed with cryptococcal antiserum; *Blastomyces* and *Coccidioides* antisera, on the other hand, did react with this antigen. This phenomenon of cross reaction between *H. capsulatum* and other fungal antisera has been observed earlier by others (1, 7, 10, 13) and is being further investigated in our laboratory.

Comments

Treatment of *H. capsulatum* (yeast phase) with pyridine provides a non-viable, workable antigen. The method has several advantages over those in use now: it is simple, is not time consuming, and does not require special equipment. The antigen obtained by this method is quite potent; it is stable for at least four months and is not anticomplementary.

On testing miscellaneous sera, this antigen displayed a high degree of specificity. On the other hand, like the antigens prepared by other methods, this crude antigen was found to react with *Blastomyces* and *Coccidioides* antisera. This serious disadvantage, however, can be overcome in the laboratory diagnosis by titrating positive reacting sera against these related antigens, since it has been shown (2) that a given serum has a higher titer with homologous than with heterologous antigen.

Work not reported here suggests that treatment with pyridine does not have detrimental effect on various antigenic components of a number of micro-organisms studied and therefore this method may yet find a wider application.

References

1. BUNNELL, I. L. and FURCOLOW, M. L. A report on ten proved cases of histoplasmosis. Public Health Repts. (U.S.), 63 : 299-315. 1948.
2. CAMPBELL, C. C. and SASLOW, S. The use of yeast phase antigens in a complement fixation test for histoplasmosis. II. Results with ground antigens. J. Lab. Clin. Med. 33 : 1207-1212. 1948.
3. CAMPBELL, C. C. Antigenic fractions of *Histoplasma capsulatum*. Am. J. Public Health, 43 : 712-717. 1953.

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4. CAMPBELL, C. C. and SASLOW, S. Use of yeast-phase antigens in a complement fixation test for histoplasmosis. III. Preliminary results with human sera. Public Health Repts. (U.S.), 64 : 551-560. 1949.

5. FISCHER, J. B. and LABZOFFSKY, N. A. Preparation of complement fixing antigen from *Cryptococcus neoformans*. Preliminary report. Can. J. Microbiol. 1 : 451-454. 1955.

6. FURCOLOW, M. I., BUNNELL, I. L., and TENENBERG, D. J. A complement fixation test for histoplasmosis. II. Preliminary results with human sera. Public Health Repts. (U.S.), 63 : 169-173. 1948.

7. HOWELL, A. Studies of fungus antigens. I. Quantitative studies of cross reactions between histoplasmin and blastomycin in guinea pigs. Public Health Repts. (U.S.), 62 : 631-651. 1947.

8. PRIOR, I. A., SASLOW, S., and COLE, C. R. Experiences with histoplasmosis. Ann. Internal Med. 40 : 221-244. 1954.

9. SALVIN, S. B. Complement fixation studies in experimental histoplasmosis. Proc. Soc. Exptl. Biol. Med. 66 : 342-345. 1947.

10. SASLOW, S. and CAMPBELL, C. C. The use of yeast phase antigens in a complement fixation test for histoplasmosis. I. Preliminary results with rabbit sera. J. Lab. Clin. Med. 33 : 811-818. 1948.

11. SASLOW, S. and CAMPBELL, C. C. Serologic studies in histoplasmosis. Am. J. Public Health, 40 : 427-435. 1950.

12. SCHUBERT, J., AJELLO, L., STANFORD, S., and GRANT, V. Q. Variation in complement fixation antigen production by different strains of *Histoplasma capsulatum* grown on two media. J. Lab. Clin. Med. 41 : 91-97. 1953.

13. TENENBERG, D. J. and HOWELL, A. A complement fixation test for histoplasmosis. I. Technic & preliminary results on animal sera. Public Health Repts. (U.S.), 63 : 163-168. 1948.

THE PHOSPHORUS FRACTIONS OF *BACILLUS CEREUS* AND *BACILLUS MEGATERIUM*

II. A CORRELATION OF THE CHEMICAL WITH THE CYTOLOGICAL CHANGES OCCURRING DURING SPORE GERMINATION[1]

By P. C. Fitz-James[2]

Abstract

The oxygen uptake, the changes in the concentrations of the phosphorus (P) fractions, and the dry weights of *Bacillus cereus* and *B. megaterium* germinating in thick suspension were followed from the spore to the young vegetative cell. Parallel cytological studies were made using standard procedures of bacterial cytology. During the initial minutes of germination the dry weight of the spores fell, respiratory activity began, the concentration of cold trichloroacetic acid (TCA)-soluble P rose, and the concentration of a residual P fraction insoluble in hot TCA fell. In complete media, nucleic acid synthesis began soon after this initial activation and was accompanied by an uptake of P, a further rise in the acid-soluble P, and in the rate of respiration. The cells began to recover weight. Ribosenucleic acid (RNA) synthesis was detectable by about 10 min. after inoculation and desoxyribosenucleic acid (DNA) synthesis by 15-20 min. Following its initial rise, the rate of RNA synthesis declined and continued parallel to that of DNA for some 10 min. During this period, the uptake of P from the medium appeared to be depressed and the spores (*B. cereus*) changed in shape from ovoids to short rods. After this period, the RNA synthesis was steady throughout germination. The rise of DNA, on the other hand, was continuous and steady throughout, even in cultures where growth was synchronous. The nuclear material of germinating spores grew and separated in step with the continuous rise of DNA P and the increase in cell volume was of the same order as the increase in RNA P. Under crowded conditions, or in inadequate media, germinating spores and young vegetative cells of *B. cereus* showed a decreased RNA/DNA ratio and accumulations of labile P. Crowded cultures of *B. megaterium*, on the other hand, accumulated Sudan positive (fatty) granules, but no labile P. The time required for the germinating spore to duplicate its chromatin varied in different media and could, in some instances, be shortened by subculturing. Nevertheless, the product of this time of germination and the increase in RNA during the germination period approximated to a fixed value that was independent of medium, species, or cell concentration.

Introduction

In the preceding paper, the concentrations of the different phosphorus (P) fractions in the resting spores and fully developed vegetative cells of *Bacillus cereus* and *Bacillus megaterium* were estimated and compared. The present paper describes the changes occurring in these fractions as resting spores develop into young vegetative cells (i.e. germinate).

A requirement for this study was to obtain thick cultures in which all cells were growing and dividing in unison. Such synchronously growing spores would be an ideal system in which to correlate the changes in the concentrations of the phosphorus fractions with the changes in morphology. Usually it is difficult to obtain bacterial cultures in which all cells are growing and dividing in unison (20). However, Powell (16) has shown that quite

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thick suspensions of spores will undergo the initial process of germination in a reasonably short interval (15 min.) when brought into contact with certain starting factors. In this study thick cultures (10^9 spores/ml.) were obtained in which over 90% of the spores had begun to germinate within four minutes of each other.

The process, referred to here as "germination", can be divided into two parts: the initial activation of the resting spore into a respiring cell, and the period of growth leading up to the formation of a young vegetative bacillus. The term "germination" is used in a more restricted sense by some workers (7, 16) to describe only the initial changes, while Preuner (19) has suggested the term "pre-vegetative" for the period of development leading up to the vegetative form. Robinow (23), taking the wide view of germination, has described the morphological changes occurring as resting spores develop into vegetative cells. He showed that basophilic areas corresponding to cytoplasmic and nuclear material were rapidly built up as the spores grew. During this development, the Feulgen-positive material of the spore (chromatin), which appeared to represent a single bacterial nucleus, became rearranged, grew, and divided, so that the young vegetative cell seen at the end of the germination contained two recently separated nuclear structures. This description prompted the present study of the changes in the phosphorus fractions throughout the over-all period of germination, particularly the changes in ribosenucleic acid (RNA) and desoxyribosenucleic acid (DNA).

Materials and Methods

Suspensions of *B. cereus* and *B. megaterium* spores were prepared as described in the previous paper.

Trial studies of the commencement of germination were done with both solid and fluid media. The ability of the spores to germinate rapidly was tested by directly observing a coverslip smear overlying a small block of heart infusion agar (Difco). The percentage of spores showing the loss of refractility and the swelling characteristic of the initiation of germination was plotted against the time elapsing from initial contact with the agar. Under controlled conditions, the 50% germination time (i.e. when half the spores had become non-refractile) could be duplicated within half a minute. In keeping with the findings of Evans and Curran (4) most batches of well-washed spores of both species began to germinate sooner after inoculation if pretreated by heating at 85° C. for 10 min. A typical effect of heat activation on the commencement of germination in *B. cereus* is shown in Fig. 1. The steeper the commencement curve, the more synchronous was the subsequent growth of the initiated spores. Microscopic examination of heated and unheated spores indicated that once the initial change of germination had begun, both forms proceeded towards the vegetative stage at a similar rate. Aging of spores at room temperature for some two to four days also improved their ability to commence germination (Fig. 1; also (16)).

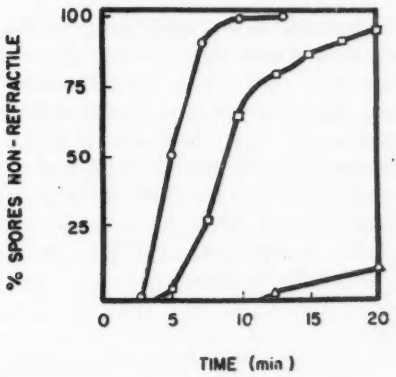


FIG. 1. Effect of aging and heat activation (85° C. for 10 min.) on the commencement of germination of *B. cereus* spores measured as percentage loss of refractility against time from initial contact with medium (heart infusion agar Difco). △————△ Freshly harvested and washed spores. □————□ After aging four days at room temperature. ○————○ After heat activation, with or without aging.

Media

After heat-treatment spores were rewashed twice in the centrifuge and suspended in water (20–30 mgm./ml.) for inoculation into one of the following types of medium:

1. *Non-synthetic or standard medium.*—Heart infusion broth (Difco) was chosen as a standard for the comparison of other media, since it was found to support readily the rapid growth of thick suspensions of spores (1–2 × 10^9 spores/ml. of culture). Its high phosphorus content, however, interfered with the accurate estimation of P uptake.

2. *Initiation medium.*—The ability of dilute solutions of L-alanine with adenosine (and, in some instances, other amino acids) to abolish both the heat stability and the refractility, as well as to initiate the respiration of resting bacillus spores is well established (7, 16, 28). However, further development of the spore does not occur in these solutions, even when glucose and phosphate are added. Hills (7) showed that the addition of other nutrilites (protein) was necessary for the subsequent growth of the activated spore. The *B. cereus* and *B. megaterium* used in this study rapidly commenced germination in solutions containing both L-alanine and adenosine. The changes in the P fractions and nuclear morphology induced by such a simple medium were studied.

3. *Semisynthetic media.*—An ideal medium for the projected studies would possess: (1) the ability to induce simultaneous germination of thick spore suspensions (10^9 spores/ml.); (2) sufficient nutrients to support the growth of the initiated spore into a vegetative cell; and (3) a P content low enough to permit a reasonably accurate estimate of changes in the concentration of P during germination. The first requirement was fulfilled by the use of

adenosine and L-alanine; the second and third by the addition of glucose and protein hydrolyzates, which were low in phosphorus. Two types of semisynthetic medium were used, which differed mainly in the origin of the protein hydrolyzate. The first medium (semisynthetic medium type A), SSA, contained adenosine (35 μM.), L-alanine (5 mM.), glucose (100 mM.), phosphate (final concentration 40 μgm. P/ml.), and casein digest (1% w/v technical casamino acids, Difco). The other medium (semisynthetic medium type B), SSB, contained, in addition to the starting constituents, glucose and enzymatic hydrolyzates of lactalbumin (1%), and yeast (0.1%). The final P content was within 30–40 μgm./ml.

Culturing Techniques

In order to obtain sufficient cells from a convenient volume of culture, reasonably high concentrations were used as the inoculum. The ability of each fluid medium to support germination and the highest concentration of spores that would rapidly commence germination in that medium were estimated by observing stained smears of such cultures. Germinating spores stained; resting spores remained refractile. These trials indicated that the initial changes of germination could be readily brought about, in thick suspensions, by increasing the concentration of one or the other of the two starting factors (usually L-alanine). However, in spite of a rapid initiation of germination, spores in thick suspensions sometimes showed a slow or abnormal growth into vegetative cells unless subcultured. Subculturing was done by mixing an aliquot of thick culture (100-200 ml.) with five to six times its volume of fresh medium of the same composition and at the same temperature as the original culture. When this step was found necessary the changes in the P fractions were studied in both the original, concentrated series (*A*) and the subcultured series (*B* and *C*).

Two methods were used for obtaining bulk germination cultures. In the first, or 'large batch' method, 1–2 liters of medium at 37° C. were inoculated with a previously established amount of spores suspended in water at the same temperature, and rocked in a 37° C. water bath. Foaming was prevented by the addition of one to two drops of tri-N-butyl citrate. Duplicate samples (20–25 ml.) for each of the two phosphorus fractionation procedures (four in all) were pipetted off at 5 or 10 min. intervals and quickly layered over 20 ml. of frozen 0.1 *N* sodium bicarbonate (tubes and buffer at 0 to − 4° C.) in a 50-ml. cellulose acetate centrifuge tube. During the subsequent centrifugation (9000 *g* for two minutes at 2–4° C.), the bicarbonate-ice melted and the sedimenting cells were rinsed. The cold residue was drained, resuspended in ice-cold 10% trichloroacetic acid (TCA), and the fractionation of phosphorus begun with the removal of acid-soluble P. Separate samples (5–10 ml.) were withdrawn at the same time intervals for the determination of medium P and cell dry weight. These samples were centrifuged in the cold and the clear supernatants analyzed for total and inorganic P. The sedimented cells were washed with cold 1% formaldehyde and rinsed into

tared weighing bottles for dry weight determination as described in the preceding publication. Occasionally, cell counts were done on a suspended portion of the washed pellet.

In the second, or 'small batch' method of handling germinating spores, an accurately measured volume (120 or 150 ml.) of medium in a 500-ml. flask was warmed to 37° C., inoculated with 2 ml. of a spore suspension (80–100 mgm.), and the flask shaken at 37° C. At the desired time period, the flask was cooled in a liquid nitrogen or dry-ice alcohol bath and the entire culture spun in steel cups on a cold, high-speed centrifuge. The pellets were washed with ice-cold alcohol; the cells resuspended in absolute alcohol and then boiled for 5–10 min. These alcohol-fixed cells suspended in alcohol were divided into four equal samples, two of which were fractionated by the Schneider (27) and two by the Schmidt and Thannhauser (26) method. For disintegration, the fixed cells were transferred to the Mickle disintegrator and disrupted as described in the preceding paper. The fractionation of the phosphorus of cells prepared by this method was begun with the lipid extraction and followed by the acid-soluble extraction.

Oxygen uptake was measured in the standard Warburg respirometer. The medium (2.5–3.0 ml.) was placed in the main chamber and a spore suspension (0.2 ml.) containing from 0.3–0.6 mgm. spores was pipetted into the sidearm. Oxygen uptake at 37° C. was measured in air at 5–10 min. intervals both before and after tipping (inoculating). The rate of oxygen uptake is recorded as the Q_{O_2} (μl. of oxygen taken up per mgm. of *resting spore weight* per hour) corrected for 100% germination.

Analytical Methods

The methods of Schneider (27) and Schmidt and Thannhauser (26) for estimating P fractions were applied as described in the previous paper. During the early period of germination, however, some substance which interfered with the estimation of DNA by the diphenylamine reaction was extracted from whole cells by hot TCA. Thus, DNA, as estimated by the method of Schneider (27) tended to rise sooner than that found by the method of Schmidt and Thannhauser (26), although subsequent increases were similar. The Racker (21) modification of the Dische (3) reaction avoided this interference, and when this was applied, the amount of DNA found by both methods of fractionation agreed closely throughout germination.

The results of the phosphorus studies on germination are expressed both as mgm. P/100 gm. of inoculated spores to give a picture of over-all synthesis and as mgm. P/weight of growing cells (or per DNA P) to illustrate the change in cell composition.

Cytological Methods

A loop of culture taken at the same time as the other samples was smeared on a small thin block of water agar (2% w/v) and immediately fixed in the

vapor of osmium tetroxide (2% w/v) for one and a half to two minutes. A transfer smear was then made by laying on a clean coverslip (#1 thickness) and flipping off the agar block. The smears were rinsed and stored in 70% alcohol. Whole and disrupted alcohol-fixed germinating spores were also smeared for cytological study.

In order to show the nuclear material, the resting and germinating spores and the vegetative cells were acid hydrolyzed and stained with basic dyes. Smears were immersed in 1 *N* HCl at 60° C. for 7–10 min. (the time was usually kept constant for any one series), rinsed with water, and stained with one of three basic stains: (*a*) Giemsa's, (*b*) thionin, reduced with thionyl chloride (SO_2-thionin) as suggested by DeLamater (2), or (*c*) azure A reduced with sodium metabisulphite (SO_2-azure A) prepared as recommended by Huebschman (8). All stained smears were washed and mounted in water. Photographs were taken with equipment previously described (5).

In one experiment in which highly synchronous growth of the germinating spores was obtained, cell and nuclear outline in the photographic negatives were projected onto filter paper sheets with a slide lantern. The outlines of the cells were then traced, cut out, and the pieces of paper measured. A similar process was then applied to the stained chromatin within the cell. The cross-sectional area and the volume of the growing cells were then calculated. Cell volume of germinating spores was calculated using the formula for a prolate spheroid $\frac{1}{6}\pi\ ab^2$ = vol., where a is the major and b the minor axis of the cell outline, and that of the vegetative cells from the volume formula of a cylinder with hemispherical ends $\frac{4}{3}\pi\ r^3 + \pi\ r^2 l$. The cross-sectional area of the nuclear structures was expressed as paper weight. At least twenty sharply focussed cells and their nuclear material were measured for each time interval.

Results

Oxygen Consumption During Germination

Well-cleaned spores of *B. cereus* took up no oxygen when in the resting state (as observed by Spencer and Powell (28), for other species), but began to respire with the first cytological indications of germination. The rate of oxygen consumption, however, varied with the medium. The response in either heart infusion broth or in the SSB medium is shown in Fig. 2. In either of these media, the spores reached the vegetative stage by 50–60 min., at which time the Q_{O_2} had reached some 400. As previously described (16) for *B. subtilis*, the spores of *B. cereus* readily lost refractility and became stainable when inoculated into a solution of adenosine and L-alanine, but their respiratory rate rose more slowly and became constant at a much lower level than was possible in more complete media (Fig. 2). Glucose (50 μM.) increased the maximum Q_{O_2} of spores respiring in such a medium, but phosphate had no effect on the respiratory rate.

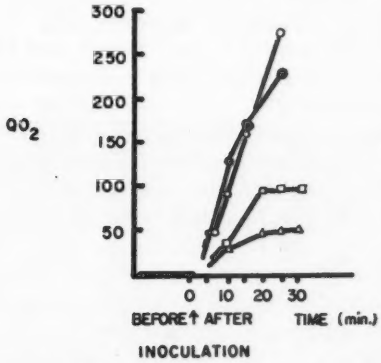


FIG. 2. Rate of oxygen uptake of *B. cereus* spores germinating in different media. ●———● In heart infusion broth (Difco). ○———○ In SSB medium. △———△ In L-alanine and adenosine without glucose. □———□ In L-alanine and adenosine with glucose.

PHOSPHORUS METABOLISM, WEIGHT CHANGES, AND CYTOLOGY OF GERMINATING SPORES

Studies with Standard Medium (Heart Infusion Broth, Difco)

B. cereus

Heat activated spores were inoculated into two liters of medium with a resulting spore concentration of 2.7 mgm./ml. Samples were taken at 0, 10, 20, 30, 40, and 60 min. (Series *A*). At 60 min., a portion was subcultured, i.e.—diluted with four volumes of fresh medium (Series *B*). The weight, P distribution, and cytology of the cells in the two series were compared at the 120 min. period. Cytological changes were followed in HCl-hydrolyzed, Giemsa-stained preparations (Fig. 3).

Between three and seven minutes after inoculation, 90% of the spores began to germinate. These non-refractile and stainable spores when hydrolyzed with acid no longer showed the one side arrangement of chromatin peculiar to acid-treated spores (23, 24). Instead, after a brief period (one to two minutes) during which the acid-hydrolyzed cells stained diffusely (22), the chromatin had assumed the position found in sectioned or disrupted resting spores appearing as an open ring or band (Fig. 3, 10 min.). In this study growth of the cells and of the contained nuclear material was first evident by 30 min. However, from this time onwards, the chromatin, when stained with Giemsa, showed little or no detail and appeared to grow as a dense granular mass which began to constrict and divide at about 60 min. This division was marked by 90 min., and complete in 90% of the cells by 120 min. and was accompanied by a change in cell outline into the shape typical of vegetative forms (Fig. 3, Series *A*). After 60 min., the growth remained reasonably synchronous, but was considerably slower than that of a subculture (Series *B*). Moreover, the cytoplasm, or perinuclear area of the 120-min. cells of Series *A* stained more deeply than was normal and

contained acid-persistent basophilic granules (Fig. 3, 120 min.). These were absent, or much less marked, in the 120-min. and 180-min. cells of Series *B*, which showed the clear non-basophilic cytoplasm more characteristic of hydrolyzed vegetative cells (Fig. 3, 120 min. sub 60 min. and 180 min. sub 60 min.). Some resistant basophilia was observable by 90 min. in Series *A*, but was not as marked as that seen later. This acid-resistant, cytoplasmic basophilia, with its attendant granules, was encountered in subsequent studies (see below) and was always associated with an increase of TCA-insoluble phosphorus.

The dry weight changes are outlined in Fig. 5. During the initial period of germination, a marked decline in the dry weight occurred similar to that described by Powell and Strange (18) for spores "germinated" in solutions of L-alanine and adenosine. By 30 min., at which time the synthesis of new cell material had become evident cytologically, the weight had risen and continued to rise steadily as the spores grew. The marked effect of subculturing, suggested by the cytology, is reflected in the dry weight increase.

In Fig. 6 the changes of acid-soluble, RNA and DNA P, are expressed on a basis of inoculated spore weight. During the first 10 min., the acid-soluble P and RNA, as determined by both methods of fractionation, showed an increase. As can be seen from Fig. 6, the RNA P at first rose rapidly, but a decline in its rate of synthesis is suggested during the 20–40 min. period, followed by a further steady increase. DNA P estimated by the Schneider method (27) using the Dische test (without the modification of Racker (21)) showed an increase by 20 min. However, when estimated by the Schmidt and Thannhauser method (26) the amount of DNA P did not begin to rise until 30 min., following which it rose steadily and had doubled the original amount by 130 min. Nuclear growth and division appeared to proceed in step with this increase, so that when 90% of the cells had reached the binucleate state (120 min., Series *A*), the DNA had increased to a similar extent (1.8-fold). The rise of DNA P was also paralleled by a rise in lipid P and in the dry weight of the cells, both of which began at about the 20-min. period.

Protein-bound, acid-labile P (Table I) was estimated in both the TCA extracts and the TCA residues of samples fractionated by the method of Schneider (27) and in the "supernatant of S & T" of those fractionated by the method of Schmidt and Thannhauser (26). During the initial 10 min. of germination, the total P of the TCA residue which contained most of the labile P of the resting spore, showed a slight fall. This fall was partly reflected by an increase in the labile P extracted by hot TCA. Following this initial change, the total P of the residues and the labile P of the TCA extracts remained constant throughout most of the germination period, but both increased slightly as the cells grew into definite vegetative forms (90 and 120 min.). Likewise in the Schmidt and Thannhauser fractionated cells an absolute increase in the amount of protein-bound labile P was found at the end of the germination period. However, both the amount of P in the TCA residue (Schneider fractionation) and the amount of labile P found via the

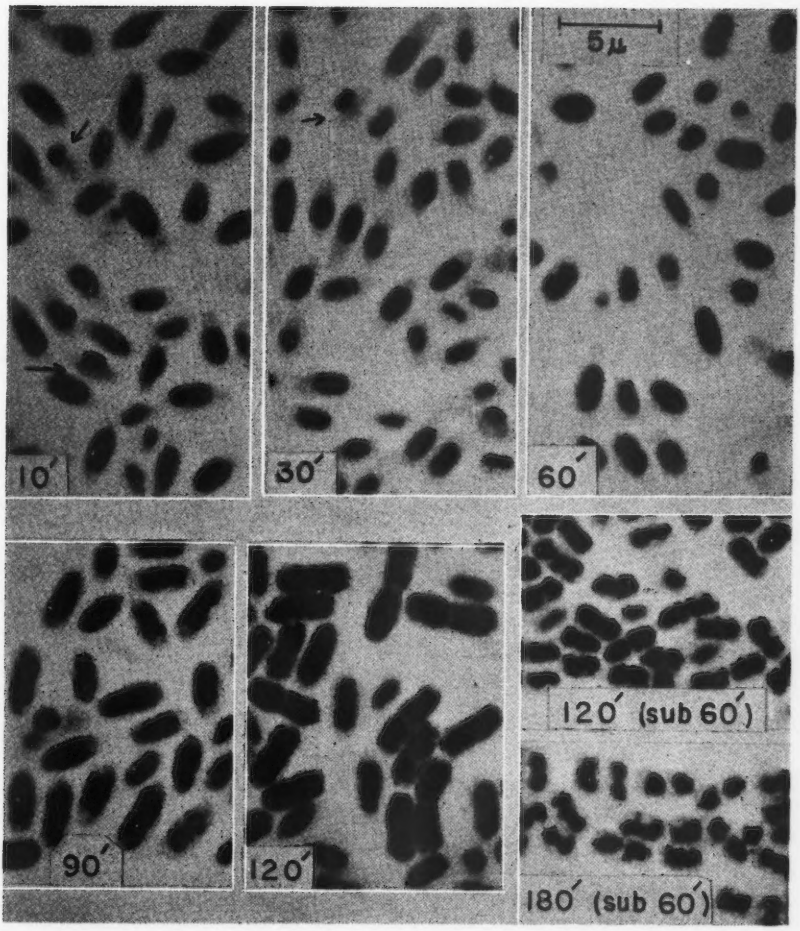


FIG. 3. Cytological changes of germination in *B. cereus* at intervals (minutes) after inoculation into nonsynthetic medium (heart infusion broth, Difco). 10-120 min. Series *A* (2.7 mgm. spores/ml.). 120 min. (sub 60 min.) and 180 min. (sub 60 min.) Series *B*—a subculture (one in five dilution) of Series *A* at 60 min. (0.54 mgm. spores/ml.). Osmium fixed, HCl-hydrolyzed preparations, stained with Giemsa. Some of the resting spores showing "peripheral nuclei" are indicated with arrows.

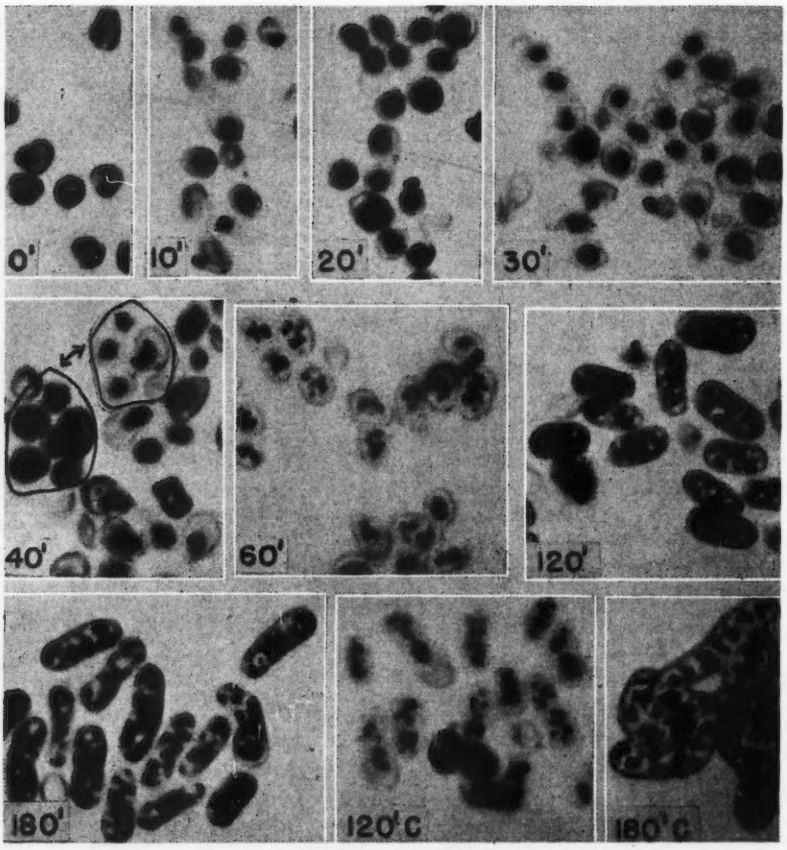
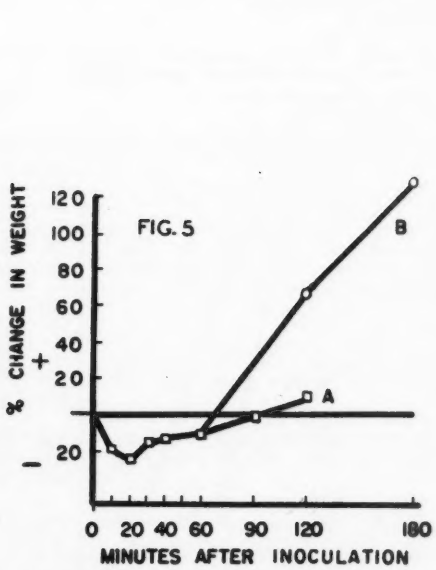


FIG. 4. Cytological changes occurring during germination of *B. megaterium* minutes after inoculation into nonsynthetic medium (heart infusion broth, Difco). 0-180 min.—Series *A* (1.37 mgm. spores/ml.). 120-180 min. C.—Series *C* a subculture of Series *A* at 90 min. (0.23 mgm. spores/ml.). All are osmium fixed, HCl hydrolyzed preparations stained with SO_2 thionin (after DeLamater, (2)). The 30 min. picture and the insert of 40 min. were lightly printed in an attempt to show the chromatin detail which is otherwise obscured by acid-resistant basophilia from 20–40 min.



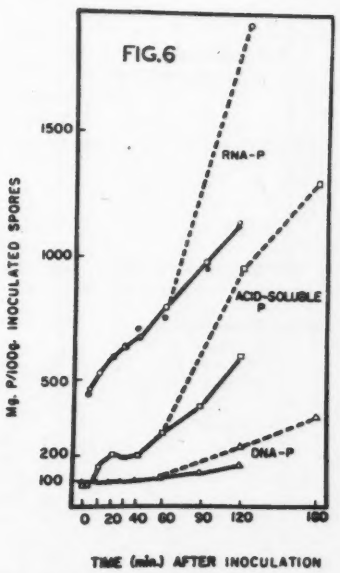


FIG. 5. Percentage change in weight of *B. cereus* germinating in heart infusion broth. □————□ A. Spore concentration 2.7 mgm./ml. ○————○ B. Subcultured at 60 min. Spore concentration 0.54 mgm./ml.

FIG. 6. The increase of RNA, DNA, and acid-soluble P of *B. cereus* spores germinating in heart infusion broth (Difco). Series *A* (solid lines) the original thick culture (2.7 mgm. spores/ml.). Series *B* (dotted lines) subcultured at 60 min. (0.54 mgm. spores/ml.). ○= = =○ RNA P (method of Schneider (27)). ● RNA P (method of Schmidt and Thannhauser (26)). □= = =□ Acid-soluble P. △= = =△ DNA P (method of Schmidt and Thannhauser (26)).

Schmidt and Thannhauser fractionation declined slightly throughout germination when expressed on the basis of cell weight or DNA P.

In Table I it can also be seen that the subcultured cells of Series *B* (120 min.) contained slightly more total labile P than those of Series *A*. Moreover, a marked difference in the distribution of labile P in these two cell groups is evident. On the basis of DNA P the 120-min. cells of Series *A* contained three times as much labile P which was insoluble in hot TCA as did the subcultured cells. The basophilic cytoplasm observed above in the corresponding HCl-Giemsa preparations might be related to the greater content of this TCA-insoluble P.

B. megaterium

Changes somewhat similar to those reported for *B. cereus* were observed when *B. megaterium* spores were inoculated into heart infusion broth (Difco). In Series *A*, the spore concentration was 1.37 mgm./ml. The effects of subculturing (one in six dilution) into the same medium at 30 min. (Series *B*) and 90 min. (Series *C*) were compared with the original culture. Some of the cytological results are shown in Fig. 4, the weight changes in Fig. 7, and the P results in Fig. 8.

TABLE I

THE DISTRIBUTION OF PROTEIN-BOUND LABILE P IN THE GERMINATING SPORES AND YOUNG VEGETATIVE CELLS OF *B. cereus* (HEART INFUSION BROTH, DIFCO)

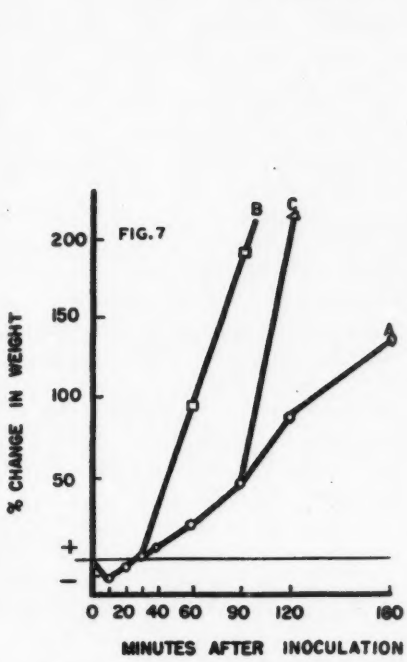
a = mgm. P/100 gm. inoculated spores.
b = μgm. P/100 μgm. DNA P.

Time (min.) after inoculation		Series *A* Pre-inoc.	0–2 min.	10 min.	20 min.	30 min.	40 min.	60 min.	90 min.	120 min.	Series *B* subcultured at 60 min. 120 min.	180 min.
Method of Schneider (27)												
TCA extracts												
Total labile P (including ortho-P)	*a*	36	57	59		51	—	50	73	117	199	193
	b	33	52	54	72	35	—	36	46	53	69	39
TCA residue												
Total P	*a*	157	138	121	121	122	122	124	140	152	128	174
	b	144	135	111	97	86	93	89	89	69	52	48
Labile P	*a*	72	74	74		78		85	95	94	43	62
	b	67	68	68		55		61	60	54	17	17
*"Supernatant of S&T"**												
Total labile P	*a*	92	103		153	136	122	136	131	196	293	310
	b	93	104		156	135	115	117	99	119	125	85

* *Method of Schmidt and Thannhauser (26) as modified in the previous paper.*

The nuclear growth of these cultures was followed in HCl-hydrolyzed preparations stained with SO_2-thionin. The resting spores of *B. megaterium* differ slightly in structure from those of *B. cereus*. They lack the exosporium of the *B. cereus* varieties and possess a non-basophilic cortical zone between the band of chromatin and the spore coat (25). By 10 min. (Fig. 4, 10 min.), some 70% of the spores possessed chromatin in the band or ring configuration characteristic of early germination. Then during the 20–30 min. period, the nuclear detail became obscured by an acid-resistant basophilia (Fig. 4, 20 min.). This coincided with the early synthesis of nucleic acids, particularly of RNA (Fig. 8), and the beginning of the weight rise (Fig. 7). By 30 min., the obscuring basophilia was most marked, but only now was the DNA significantly elevated. Granules contributed to the dense material but they were somewhat obscured by halation in the photomicrographs (Fig. 4, 30 min.). At 40 min., most cells were shedding their coats and revealed after hydrolysis nuclear detail which could be more clearly photographed. By 60 min., the granular network of the growing chromatin was clearly visible and by 90 min., the separation of the granules into two groups was seen in most cells. By this time the DNA, having commenced its rise between 20 and 30 min., had increased by a factor of two (Fig. 8).

Subculturing also had a marked effect on the cytology of the early vegetative cells of this species. The nuclear material of the 120- and 180-min. cells of



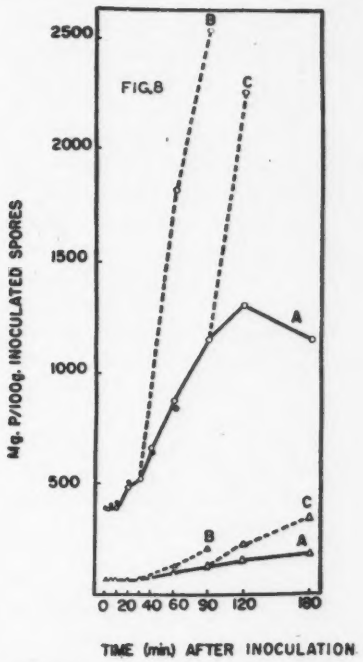


FIG. 7. Percentage change in weight during germination of *B. megaterium* in heart infusion broth (Difco). Cell concentration (original spores mgm./ml.): A, 1.37; B and C, 0.73.

FIG. 8. The RNA P and DNA P of *B. megaterium* spores germinating in heart infusion broth. Series *A* (solid lines), the original thick culture (1.37 mgm. spores/ml.) and Series *B* and *C* (dotted lines) subcultured at 30 and 90 min. respectively (0.23 mgm. spores/ml.). Calculations based on weight of inoculated spores. ○═══○ RNA P (method of Schneider (27)). ● RNA P (method of Schmidt and Thannhauser (26)). △═══△ DNA P (method of Schmidt and Thannhauser (26)).

the original thick culture (Series *A*) appeared to be stretched about, or intermixed with, clear vacuole-like areas (Fig. 4, 120- and 180-min. cells). Cells grown from diluted cultures showed, at the same time period (Fig. 4, 120 min., C), the typical picture of chromatin strands, and granules unencumbered by these refractile bodies. Yet, by 180 min., the subculture itself became crowded and the internuclear refractile zones reappeared (Fig. 4, 180 min., C).

These observations prompted a comparison of the cytology of *B. megaterium* cells at the later stages of germination or early vegetative stage in both thick and dilute cultures. Both nuclear (HCl-Giemsa and HCl-azure A) and fat (Sudan black) stains were used. In thick cultures (1–2 mgm. spores/ml.), Sudan-positive granules appeared within two to three hours of spore inoculation, coincident with the appearance of refractile bodies in the HCl-hydrolyzed preparations similar to those in Fig. 4. These inclusions were first seen as fine Sudan-positive areas closely associated with the chromatin

structures. The cells of the dilute cultures (0.2-0.4 mgm./ml.) did not show these refractile bodies until some two to three hours later.

The production of a fatty substance (a polymer of β-hydroxybutyric acid) by vegetative cells of *B. megaterium* is well known (9, 29), and presumably this same material is responsible for the Sudan staining granules seen closely associated with the chromatin of cells growing in crowded cultures only two hours after spore inoculation.

The fall of weight on the initiation of germination of this species (Fig. 7) was also followed by a steady rise, and this could be greatly accelerated by subculturing. The break in the rise of RNA P observed above for *B. cereus* during the period of early nuclear growth and DNA rise (30–40 min.) was also found to occur during the germination of *B. megaterium* but somewhat earlier (20 and 30 min.). From Fig. 8 it can be seen that subculturing had a marked effect on the synthesis of both nucleic acids, but particularly of RNA (Table VII) and that conditions for continued growth in the thick cultures were far from optimal. In fact, in the original culture, a decline in the rate of DNA synthesis and a fall in the amount of RNA P estimated by the orcinol reaction had occurred by 120 min.

The DNA P, when calculated as a percentage of the dry weight of the growing cells, was remarkably constant throughout germination and in the unsubcultured (crowded) vegetative cells. In the more vigorously growing (subcultured) cells, however, the DNA P/dry weight of cells was some 18–20% lower.

The large amount of P in the spore coat residue ("residue of S&T") of *B. megaterium* remained constant throughout germination. The TCA residue (Schneider method, 27) which includes both spore-coat P as well as some other P material, possibly acid-labile in nature, showed a slight drop (5%) during the initial 30 min. of germination.

Studies with Initiation Medium

When *B. cereus* spores were inoculated into the synthetic medium of L-alanine and adenosine in bicarbonate buffer, used by others to study the initial changes of germinating spores (16, 28, 18), they rapidly lost refractility, became stainable, and began to respire. The initial drop in dry weight observed by Powell and Strange (18) was confirmed and, in contrast to spores germinating in the more complete standard medium, there was no weight recovery. Instead, the dry weight continued to fall. Phosphorus fractionation of such initiated spores by the method of Schneider (27) showed no synthesis of either nucleic acid and the RNA/DNA ratio remained constant throughout a two-hour period. However, coincident with the early loss of refractility, there was an increase in acid-soluble P, which could only be accounted for by a slight drop in TCA-residue P. These changes in acid-soluble and residue P were of the same order as those shown in Table III from 0–20 min. In spite of the fairly active respiration (Fig. 2), added phosphorus was not taken up from the medium by these activated, but non-growing spores. The cytological changes were similar to the initial changes,

up to 20 min. shown in Fig. 9 (5, 10, and 20 min.). No evidence of cellular or nuclear growth was observed, even two hours after inoculation. Instead, by then, many of the spore protoplasts, when stained without hydrolysis, appeared to be distorted and partially collapsed while after hydrolysis the nuclear bands were still in the resting configuration. These cytological pictures together with lack of nucleic acid synthesis suggested that, on prolonged incubation in initiation medium, the respiring spores were not growing, but were utilizing their own substance.

Studies with Semisynthetic Medium Type A (SSA)

B. cereus

When casein hydrolyzate (casamino acids, technical, Difco) was used to supplement the starting factors (SSA medium), active growth with rapid synthesis of nucleic acids (as found above for the standard medium) proceeded only in the dilute subcultures. In thick cultures, both the weight response and the cytological development reflected the poor nucleic acid synthesis. However, interesting structural and chemical changes occurred when thick suspensions of germinating *B. cereus* were maintained in SSA medium and for this reason a typical study is outlined.

Heat activated *B. cereus* spores were inoculated into a 'large batch' of medium containing adenosine, L-alanine, glucose, and 'casamino' acids in sodium bicarbonate containing 40 μgm. P/ml. The final spore concentration was 2.03 mgm./ml. (2×10^9 spores/ml.). The cytological results are shown in Fig. 9, the weight changes in Table II, and the phosphorus results, in part, in Table III.

As in the standard medium, 90% of the spores had begun to germinate within five minutes. After acid hydrolysis they again showed a period of diffuse staining characteristic of the initiation stage in this species (22). (Note, in contrast, the two resting spores in Fig. 9, 5 min., with chromatin displaced as a side body). At 10 and 20 min., however, where one would normally expect to see the beginning of nuclear growth, the chromatin was still in the predivisional arrangement—a beaded band or claw, gripping the

TABLE II

PERCENTAGE CHANGE IN DRY WEIGHT OF *B. cereus* SPORES GERMINATING IN SEMISYNTHETIC MEDIUM, SSA

Time after inoculation	% change in weight	
	Expt. *a*	Expt. *b*
10 min.	− 10.1	− 21
20 min.	—	− 21
30 min.	− 4.2	—
40 min.	− 4.2	− 21
60 min.	− 0.1	− 12
4 hr.	− 19	—
4 hr. subcultured at 60 min.	+ 353	—

TABLE III

THE PHOSPHORUS FRACTIONS OF *B. cereus* GERMINATING IN SEMISYNTHETIC MEDIUM, SSA

0–60 min., mgm. P/100 gm. resting spores
4 hr. cells, mgm. P/100 gm. cells

Time (min.) after inoculation	Control 0 min.	2½ min.	5 min.	10 min.	15 min.	20 min.	30 min.	40 min.	60 min.	Four hours: Thick culture	Four hours: Subcultured at 60 min.
Medium P	2100	2130	—	2058	—	2018	1931	—	—	—	—
Acid-soluble P	139	175	180	200	—	—	—	—	—	—	—
RNA P (orcinol)		383	382	387	396	396	410	430	477	720	1000
DNA P (diphenylamine)	78	77	78	80	80	82	86	90	99	127	162
Residue P (Schneider (27))	120	117	96	100	107	112	126	144	161	190	45
"Supernatant of S&T"* Total labile P	60	—	48	40	41	—	—	—	140	300	105

* *See footnote, Table I.*

RNA-containing mass of the spore. Thus, although germination began readily in this medium, it did not proceed normally. By 30 and 40 min., although a progressive increase in HCl-resistant basophilia was observed, it was not uniform in all cells and not characteristic of normally developing chromatin (Fig. 9, 30 min. and 40 min.). At 60 min., this disparity was even greater. A few cells showed the beginning of nuclear growth, but in most it remained arrested at the 20-min. stage. By four hours, some of the cells had developed into vegetative forms but, as no gain in dry weight was observed (Table II), one would suspect that vegetation in some had been at the expense of lysis in others. Moreover, these vegetative cells from the thick culture showed the same accumulation of basophilic substances observed above in the standard medium study. Here, however, the accumulation was much more marked, and often appeared as granules obscuring the chromatin structures. The staining response of acid-hydrolyzed cells grown four hours from a subculture was normal. (Fig. 9, compare four hours and four hours sub 60 min.).

The decline of medium P shown in Table III indicates that the spores assimilated P. The acid-soluble P showed the rise previously associated with the initiation of germination and the RNA P began to rise 10 min. after inoculation. DNA synthesis appeared to be poor, having increased only 1.25-fold by 60 min. At this time, a slight weight recovery was recorded. In the light of the cytological findings, the most interesting changes observed in this experiment were those of the residue P (method of Schneider (27)). After an initial fall during the first 5–10 min. of germination, the amount of this P increased (Table III). Labile P studies on this and a repeat experiment showed that the labile P content of this TCA residue throughout the period of rise (30–60 min. after inoculation) equalled the total amount of protein-bound labile P found by the fractionation method of Schmidt and

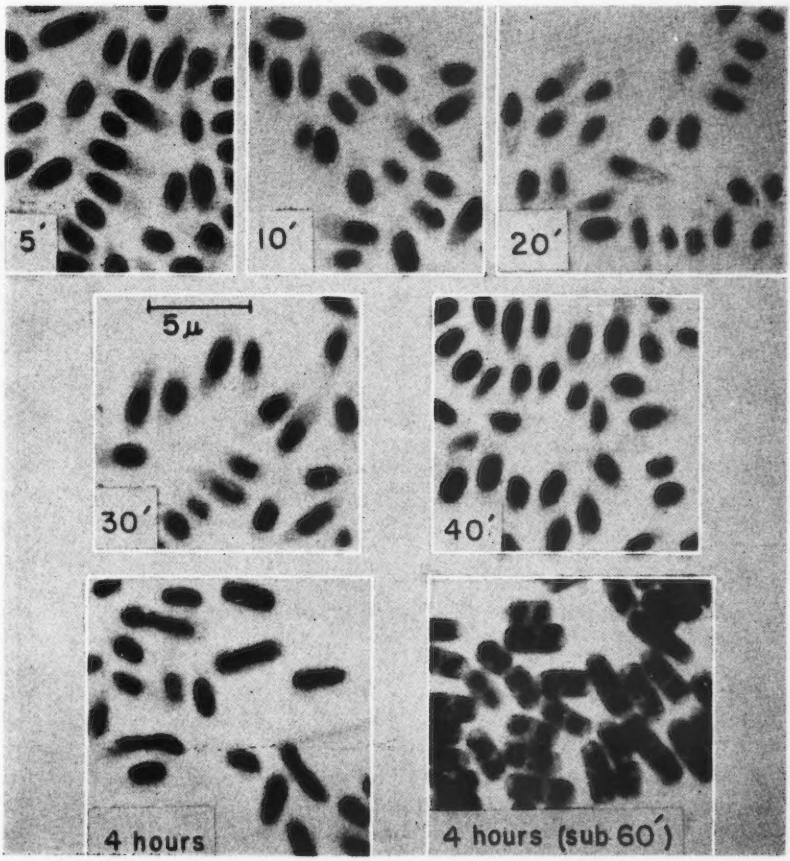


FIG. 9. The cytological changes in *B. cereus* spores at different times (minutes) following inoculation into a somewhat inadequate germination medium (SSA). 5-40 min.—changes in thick cultures 2.03 mgm./ml. 4 hours—appearance of abnormal vegetative cells developed in the thick cultures. 4 hours (sub 60)—more normal vegetative cells grown from a subculture (1/6 dilution) into fresh medium at 60 min. Osmium fixed, *N* HCl hydrolyzed (8–10 min. at 60° C.) Giemsa stained.

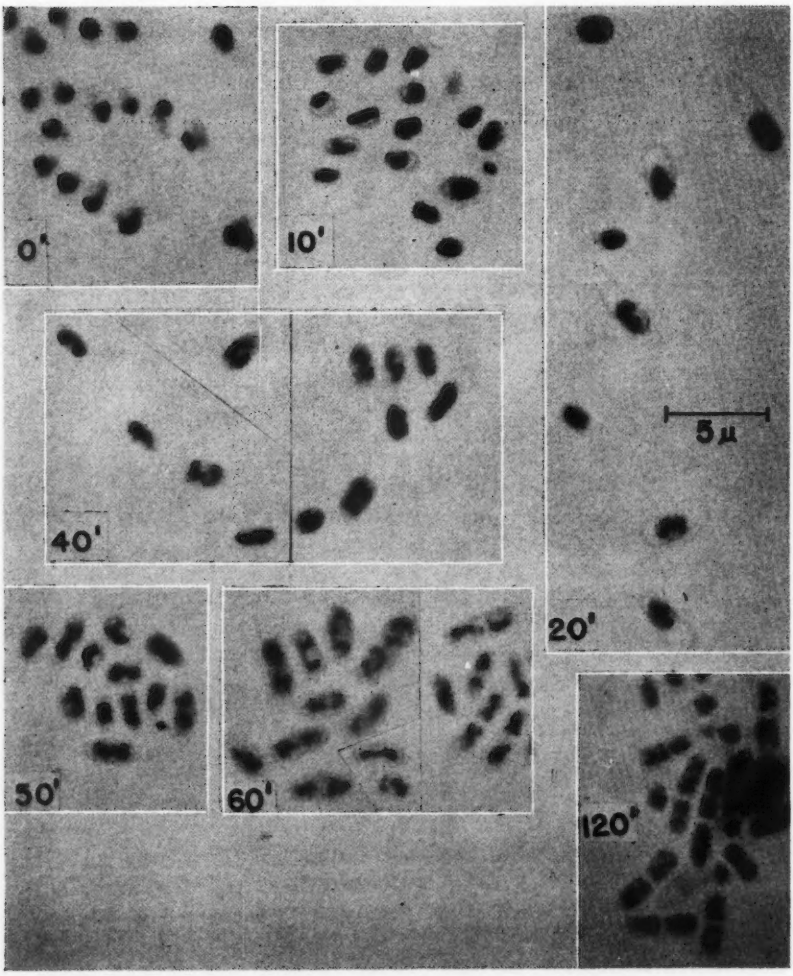


FIG. 10. The chromatin of germinating *B. cereus* spores fixed with osmium, hydrolyzed with *N* HCl (60° C., 8–10 min.) and stained with SO_2-azure A. Magnification as indicated.

Thannhauser (26). This suggested that most of the newly formed labile P was insoluble in hot TCA. By four hours, the cells of the thick culture contained much less RNA P (but relatively more labile P) than those of the subculture. Some 29% of the protein-bound P of the crowded and abnormal cells (four hours, Fig. 9) was acid-labile, compared to only 8% for the normal ones. Moreover, 19% of the entire protein-bound P of the abnormal cells was in a TCA-insoluble form, compared to some 3–4% for the subcultured cells (Table III). Thus, while nearly all the protein-bound labile P of the typical vegetative cells was extracted by hot TCA, that of the atypical ones was found half in the TCA extracts and half in the TCA residue (method of Schneider (27)).

The nature of the labile P in these cells has not been established but is assumed to be similar to the labile P material found in (or on) the resting spores of this species and shown to exhibit some of the properties of polymetaphosphate (see previous paper). It again seems reasonable to suppose that the high content of TCA-insoluble P is directly related to the basophilic substance which persists in the four hour cells after HCl hydrolysis (Fig. 9).

The estimation of nucleic acid by the ultraviolet absorption of the TCA extracts (after Logan *et al.* (10)) was not usually possible with whole resting spores, since an interfering substance gave abnormally high values for the nucleic acid P. Once spores had commenced to germinate, however, the nucleic acid by the ultraviolet method agreed well with the total nucleic acid P as estimated by other methods. In a separate experiment, the ultraviolet absorption spectrum of a semisynthetic medium was examined after it had supported germination for 5, 10, 20, 30, and 60 min. Some 90% of the spores had begun to germinate by five minutes. The ultraviolet absorption of the five-minute medium, read against a medium blank is shown in Fig. 11. The figure shows the absorption curve of the interfering substance encountered in the TCA extracts of resting spores (see previous paper). No changes, quantitative or qualitative, in spectrum were observed in the subsequent samples of germination medium (up to 60 min.) indicating the early release of this interfering substance from the germinating spores. Since these studies were made, the work of Powell and Strange (18) has appeared, reporting a similar ultraviolet absorption spectrum in germination media and establishing calcium dipicolinate as the material responsible (17).

B. megaterium spores also showed a poor growth response in the SSA medium (Table IV). Only in dilute cultures did some growth and the synthesis of nucleic acids occur. The effect of such subculturing is shown in the RNA P/DNA P ratios (Table VII). No accumulation of insoluble labile P was observed with this species during the period of poor growth. Labile P was found, however, in the cold TCA extracts of these spores and the changes during germination are recorded in Table IV. During the initiation period (i.e. before nucleic acids were synthesized) 0–10 min., a rise in acid-soluble labile P occurred. The proportion of labile to total P in the acid-soluble extracts tended to remain constant or fall slightly during the period

TABLE IV

GERMINATION OF *B. megaterium* IN SEMISYNTHETIC MEDIUM SSA
mgm. P/100 gm. inoculated spores

		"B" series (subcultured at 15 min.)							"A" series (original culture)	
	Control	0–2 min.	5 min.	10 min.	20 min.	30 min.	40 min.	60 min.	30 min.	60 min.
Uptake of ortho-P from medium (accumulative)	0	0	10	19	38	100	100	320	63	276
Acid-soluble P (ASP)										
Total P	24	35	66	101	187	213	258	323	183	260
Ortho-P	2	14	18	31	58	65	77	92	43	77
Acid-labile P (corrected for ortho-P)	1	3	7	14	34	35	41	45	43	41
as % of total ASP	4.1	8.5	10.6	13.8	18.2	16.4	15.9	13.9	23.5	15.7
RNA P*	410	400	396	396	395	447	449	604	396	462
DNA P	72	72	70	71	70	71	80	85	71	76

* *See footnote, Table I.*

of nucleic acid synthesis (30–60 min.). The results in Table IV suggest that the rise of total acid-soluble P in the initial minutes of germination is independent of the medium phosphorus. As with *B. cereus* this early increase in acid-soluble P was balanced by a fall in residue P.

Studies with Semisynthetic Medium Type B (SSB)

Heat activated *B. cereus* spores were inoculated into the medium in an initial concentration of 0.67 mgm./ml. The 'small batch' method of culturing was used and the cells were fixed with boiling alcohol.

In the results recorded above, the germinating spores were subjected to the P fractionation procedures as whole cells. Here, both whole and disrupted germinating spores were analyzed. This was done in an attempt to decide whether the fall in protein-bound labile P and the concurrent rise in acid-soluble P observed above during the initial period of germination were due to a simple change in the permeability of the spore membranes (similar to that brought about by disruption of resting spores, see previous paper), or to some biological process, whereby chemically bound P was converted to acid-soluble P.

Both the cytological and chemical findings indicated that germination was more rapid here than in the standard medium. Fig. 11 records the chromatin changes in these normally and rapidly germinating spores. With the dye system used here (SO_2-azure A) nuclear structures were stained more delicately than those treated with Giemsa's stain in the standard medium study. As early as six minutes after inoculation the majority of the spores had lost refractility and passed through the diffusely staining stage. After hydrolysis, the chromatin of these five- to six-minute spores was arranged in the open

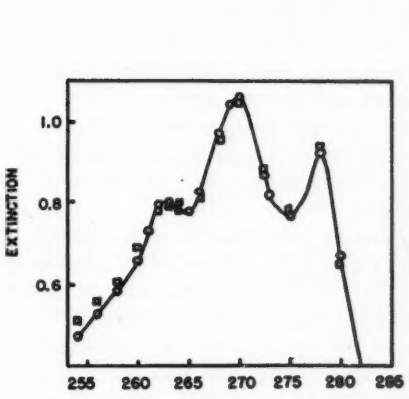


FIG. 11

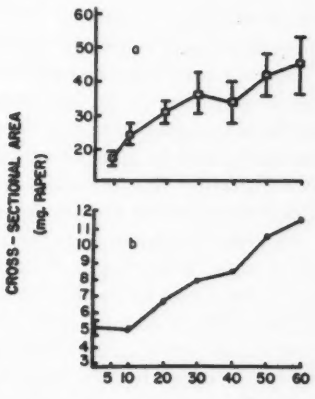


FIG. 12

FIG. 11. The ultraviolet absorption spectrum of a semisynthetic medium (SSA) having supported the germination of *B. cereus* spores (1.0 mgm./ml.) for 5 (○) and 30 min. (□). Samples were diluted with an equal volume of water and read against a similarly diluted sample of uninoculated medium.

FIG. 12. A quantitative expression of the cytology of *B. cereus* spores germinating in SSB medium. *a*, □——□ average cross-sectional area of cells (with maximum range of values). *b*, ●——● average area of chromatin per cell.

band or "claw" pattern described above. By 10 min., the chromatin had contracted into a structure resembling a triangle of granules, which can be seen in the photomicrograph from various angles.

During the subsequent growth of the spore, the chromatin increased in amount. At 50 min., it was arranged logitudinally in the short, rod-shaped cells. By 60 min., it had separated into two structures many of which showed the triangular pattern of the earlier forms; a few indicated further continued growth. Granular chromatin, arranged in patterns similar to those seen in germinating spores, was crowded in the cells of a 120-min. subculture (Fig. 10). These changes in nuclear structure were also followed by the projecting and tracing technique outlined in the methods, as were also changes in the cell outline.

The cross-sectional area of the cells increased steadily up to 30 min., declined slightly during the 30–40 min. period, then rose again (Fig. 12*a*). This 30–40 min. decline coincided with a change in dimensions of the cells. For example, the average dimensions, in arbitrary units (± S.E.M.) after 30 min., were 33.3 (± 0.83) × 23.0 (± 0.60) and by 40 min. were 36.3 (± 1.02) × 20.7 (± 0.48). An analysis of these values revealed a significant narrowing of the average cell diameter ($P < 0.01$) and a probably significant increase in cell length ($P < 0.05$). That is, during the 30–40 min. interval, the ovoid spores became rod-shaped. From the average cross-sectional dimensions of these growing cells, the volume was calculated. From 0–30 min. the volume formula for a prolate spheroid was used; after this period, that of a cylinder

with hemispherical ends. During the 60 min. of germination there was a fourfold increase in cell volume of the resting spore (1.04 μ^3 to 4.35 μ^3).

Fig. 12*b* shows the rise of the average cross-sectional area of the stainable nuclear material of the cell (expressed as arbitrary units). From 0–10 min., the cross-sectional area of chromatin showed no increase, but by 20 min. an increase in area had begun which continued throughout germination (compare with Fig. 10). These studies also established that the newly divided "nuclei" were always over a certain critical size.

Phosphorus uptake from the medium was followed in two separate studies and the Q_P values (μgm. P taken up per mgm. dry weight of resting spores/hour) are shown in Table V. The rate of P uptake showed a definite decline between 30 and 40 min. in both studies following which it increased. In both these rapid germination experiments, an extension of the P uptake curve met the origin soon after the spores first lost refractility, suggesting the onset of synthesis soon after the initial activation.

From Table VI it can be seen that although disruption before fractionation resulted in an increase in the total acid-soluble P of resting spores (0 min.),

TABLE V

Q_P VALUES OF *B. cereus* SPORES GERMINATING IN SEMISYNTHETIC MEDIUM, SSB
(Q_P = μGM./P TAKEN UP FROM MEDIUM/MGM. DRY WEIGHT OF RESTING SPORES/HOUR)

	Time intervals in minutes								
	0–5	5–10	10–15	15–20	20–30	30–40	40–50	50–60	60–120
Experiment I	12.5	17.9	19.7	21.5	18.8	15.2	23.8		—
Experiment II	16.1	19.7	21.5	19.7	19.7	16.1	34.0	43.0	19.4

TABLE VI

THE ACID-SOLUBLE AND RESIDUE-P OF WHOLE AND DISRUPTED *B. cereus* SPORES FOLLOWING GERMINATION (MIN.) IN SEMISYNTHETIC MEDIUM SSB
a = mgm. P/100 gm. inoculated spores.
b = μgm. P/100 μgm. DNA P.

		0 min.	5 min.	10 min.	20 min.	30 min.	40 min.	50 min.	60 min.
Whole cells									
Acid-soluble P	*a*	10	100	155	184	263	306	395	414
	b	10	111	163	172	273	235	269	229
TCA-residue P (Schneider, (27))	*a*	199	157	157	154	169	201	—	243
	b	220	180	163	145	144	154	—	138
"S&T supernatant"* Acid-labile P	*a*	146	153	145	132	150	170	—	180
	b	162	173	151	136	134	128	—	99
Cells disrupted before fractionation									
Acid-soluble P	*a*	50	114	157	178	—	—	—	—
	b	49	116	170	172				
TCA-residue P (Schneider, (27))	*a*	140	—	108	120	147	163	174	214
	b	134		117	123	125	120	96	80

* *See footnote, Table I.*

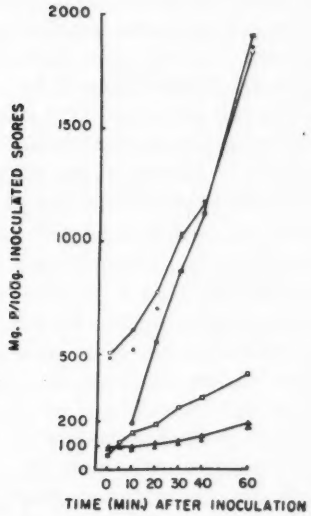


FIG. 13. The phosphorus fractions of *B. cereus* germinating in semisynthetic medium (SSB). P as mgm./100 gm. inoculated spores. ■———■ P uptake. □———□ Acid-soluble P. ○———○ RNA P (method of Schneider (27)). ● RNA P (method of Schmidt and Thannhauser (26)). △———△ DNA P (method of Schneider (21) after Racker (21)). ▲ DNA P (method of Schmidt and Thannhauser (26)).

it did not augment the already increased acid-soluble P of germinating spores. Moreover, the increase in total acid-soluble P during early germination was much greater than that brought about by rupturing resting spores. These results would lead one to conclude that the liberation of P from a bound to an acid-soluble form during the initial moments of germination is not affected by a simple change in the permeability of the spore. A fall of residue P at the onset of germination was again associated with this early rise of acid-soluble P.

Most of the changes in the P fractions previously described in germinating *B. cereus* in the standard medium were found here (Fig. 13). Again RNA was found to rise early in germination and to show a slight decline of its rate of increase during the 30–40 min. period. By 60 min. the RNA had, like the cell volume, increased fourfold. The amount of DNA started to increase during the 10–20 min. interval and had doubled that of the spore by 60 min. The slight drop in residue P occurring at the onset was followed by a rise in the later periods of germination. The total labile P also increased towards the end of germination. However, as before, both the TCA-residual P (fractionation of Schneider, (27)) and total labile P (fractionation of Schmidt and Thannhauser, (26)) declined throughout when the values were expressed on a basis of the DNA-content (or dry weight) of the cells (Table VI).

Under these more optimum conditions, subculturing at 30 min. did not accelerate nucleic acid synthesis until after some 60 min. By 120 min. a need for subculturing was observed. At this time, the cells in the original culture had increased 2.8-fold in weight, while those in the subculture had increased 6.5-fold. The difference in amount and in TCA solubility of the labile P of *B. cereus* described above was again encountered on comparing the two types of vegetative cell in this study. Moveover, the 120 min. cells of the original culture showed the acid-resistant cytoplasmic basophilia noted previously.

A correlation of the cytological and chemical data indicated some interesting relationships. During the 60 min. of germination in the SSB experiment, the DNA P had doubled, but the RNA P increased fourfold (Fig. 13). The cell volume increase was likewise fourfold in 60 min. This increase suggests that the concentration of RNA P in the cytoplasm might be constant throughout germination. A plot of the area of chromatin (as mgm. of paper) against the DNA P of the culture or, since there is no multiplication, against the DNA P per cell (Fig. 14) indicated that the DNA P throughout germination was proportional to the stainable nuclear material. A theory of the mechanism of chromatin duplication based upon the cytological and chemical evidence presented here has been published elsewhere (6).

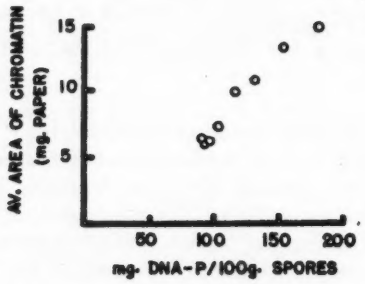


FIG. 14. The relation between basophilic nuclear material (chromatin) and DNA P during the rapid germination of *B. cereus*.

In all experiments showing a synthesis of both nucleic acids, a slowing in the rate of RNA formation was recorded shortly after the beginning of DNA synthesis. This is reflected by the ratios of RNA P to DNA P summarized in Table VII. Also shown in this table are the times required for the spores of the two species to complete the process of germination in the various media and at different cell concentrations.

The RNA content of a bacterial cell apparently indicates the rate at which it is growing (11, 1, 20) and from Table VII it can be seen that this is true for the growth of spores into young vegetative cells. Since the increase of DNA P paralleled the weight rise, the RNA P/DNA P is a reliable index of RNA

TABLE VII

A SUMMARY OF THE RNA P/DNA P RATIOS OF *B. cereus* AND *B. megaterium* GERMINATING IN VARIOUS MEDIA SHOWING THE RELATIONSHIP OF GERMINATION TIME* TO SYNTHESIS OF RNA P

Medium	Organism	Culture series	Cell concentration as inoculated spores, mgm./ml.	RNA P/DNA P during germination (time in min.)												Germination time* (min.)	RNA P synthesized during germination, mgm. P/100 gm. spores	Germ'n. time × RNA P syn. / 1000
				Resting spores	5	10	15	20	30	40	50	60	90	120	180			
Heart infusion broth (Difco)	*B. cereus*	*A*	2.7	4.6	—	5.4	—	6.1	6.4	6.4	—	6.9‡	7.4	7.4	—	120†	710	8.5
		B	0.54									6.9	—	8.5	7.8	90	930	8.4
	B. megaterium	*A*	1.37	5.4	—	6.0	—	7.6	7.6‡	8.6	—	9.7§	10.0	8.5	6.8	90	954	8.5
		B	0.23						7.6	—	—	18.0	13.5	—	—	60	1432	8.6
		C	0.23									9.7	10.0	12.1	16.3	—	—	—
Semi-synthetic medium, Type A (SSA)	*B. cereus*		2.03	4.5	4.4	4.3	4.6	4.6	4.7	5.0	—	5.3	—	—	—	Delayed	—	—
			1.14	4.6	4.3	4.5	—	5.0	5.4	5.7	—	6.4	—	8.0	10.3	germination	—	—
	B. megaterium	*A*	2.66	5.9	5.6	5.6	5.6‡	—	5.6	—	—	6.1				Delayed	—	—
		B	1.33				5.6	5.6	6.3	5.6	—	7.1				germination	—	
Semi-synthetic medium, Type B (SSB)	*B. cereus*		0.67	4.8	4.5	5.7	—	7.2	8.5	8.7	10.1	10.0				60	1400	8.4

* *The time required for the DNA P or chromatin to double the spore amount.*
† *When 90% of these cells had doubled the spore amount of DNA and chromatin.*
‡ *Subcultured to fresh medium to begin Series B.*
§ *Subcultured to fresh medium to begin Series C.*

content. The shorter the time required to reach the end of germination, the greater the RNA content at that time. Moreover, the amount of RNA synthesized during the development of a spore to a "binucleate" vegetative cell was inversely proportional to the time required. That is, the product of the germination time and the RNA P synthezsied during germination (Table VII, last column) was a reasonably constant value independent of species or medium.

Discussion

The time during which a spore changes to become a vegetative rod can be divided into a period of activation and a period of synthesis.

When placed in a medium capable of supporting both these activities, heat-treated resting spores, which had no measurable respiration, showed a sudden commencement of and a linear increase in respiratory activity. Oxygen uptake appeared to begin soon after the spores lost refractility and became swollen. It was also encountered in media capable only of initiating germination. As shown by previous work (7, 16) and the results reported here, the initiation of germination is brought on by simple organic compounds (adenosine and L-alanine) and is independent of external glucose and phosphorus. Previous heat treatment hastens the initiation.

It has previously been shown that during the initial period of germination spores become altered in their response to acid hydrolysis (24) and lose weight (18). Powell and Strange (18) suggest that with the release of solids the spores take up water. Data supporting this suggestion was obtained when ethanol-washed resting spores, germinating spores, and vegetative cells were disrupted in absolute ethanol and the water content of these extracts examined (Fitz-James and Vance, unpublished data).

The response of germinating spores in various media indicates that during the initial awakening the only change occurring in the phosphorus distribution is an increase in cold acid-soluble P, independent of medium P and coinciding with a fall in a P fraction insoluble in hot TCA. A major part of the increased acid soluble phosphorus is 7 min. acid labile. Thus, during the activation period, protein-bound phosphorus of the dormant spore becomes acid soluble.

The assimilation of phosphorus and a more marked rise in respiratory activity, characteristics of the second period of spore germination, are found only when the medium is able to support the growth of spores into vegetative cells. Within 10 min., such assimilating spores show a significant increase of RNA and a further increase of acid-soluble P. The synthesis of DNA begins some few minutes after that of RNA. The slight decline in the rate of RNA synthesis which follows the initial DNA increase coincides (in *B. cereus*) with the period when the cells are changing from spheroid to cylindrical structures. During this interval the P uptake is also decreased, but the DNA rise continues steadily. The significance of this decrease in the rate of RNA synthesis is not clear. On comparing the chromosomes of different types of fish and mammalian cells, Mirsky (12) found that the RNA content of 'residual'

chromosomes from actively metabolizing cells was higher than that from relatively inactive ones. If such a balance of nuclear RNA and cellular activity could be applied to these microorganisms, the RNA synthesized in the first 30 min. might be distinct from that formed later, and could correspond to 'nuclear' RNA, being associated with the synthesis of chromatin in the awakening cell. Following its initial rise, this RNA could remain constant in relation to the rising DNA but would be shortly overshadowed by the more rapid synthesis of 'cytoplasmic' RNA. Although direct evidence of the presence in bacteria of two such distinct types of RNA is lacking, the presence of an RNA moiety structurally associated with bacterial chromatin has already been indicated in some cytological studies (13, 25).

The constant relationship found between the amount of RNA synthesized and the time of germination further emphasizes the important role of this nucleic acid in cell synthesis.

The acid-labile residual P which accumulates during the germination and early vegetative growth of *B. cereus* under crowded conditions is presumed to be similar to the labile P found in (or on) the resting spores of this species, a material which, when brought into solution with dilute alkali, behaved in many ways like highly polymerized metaphosphate (see preceding paper). Normal rapidly dividing vegetative cells likewise contain protein-bound labile P but it is readily extracted by hot TCA. Moreover, the crowded cells, in contradistinction to the normal ones, contained accumulations of a basophilic material which resisted the Feulgen hydrolysis and did not show the usual arrangement of bacterial chromatin. This accumulation of labile P by germinating spores or young vegetative cells growing under adverse conditions suggests the work of Park and Johnston (15) who found that an acid-labile P accumulated in *Staphylococcus aureus* grown in the presence of penicillin. However, their penicillin-induced labile P is apparently cold-acid soluble and contains uridine-5-pyrophosphate (14).

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References

1. CALDWELL, P. C., MACKOR, E. L., and HINSHELWOOD, C. The ribose nucleic acid content and cell growth of *Bact. lactis aerogenes*. J. Chem. Soc. Part IV : 3151-3155. 1950.
2. DELAMATER, E. D. A staining and dehydrating procedure for the handling of microorganisms. Stain Technol. 26 : 199-204. 1951.
3. DISCHE, Z. Uber einige neue charakteristische Farbreactionen der Thymonuclein sönne und eine micromethod sur Bestimmung derselben in tierischen organen mit Hilfe dieser Reaktionen. Mikrochemie, 8 : 4–32. 1930.

4. EVANS, F. R. and CURRAN, H. R. The accelerating effect of sublethal heat on spore germination in mesophilic aerobic bacteria. J. Bacteriol. 46 : 513–523. 1943.
5. FITZ-JAMES, P. C. The structure of spores as revealed by mechanical disruption. J. Bacteriol. 66 : 312–319. 1953.
6. FITZ-JAMES, P. C. The duplication of bacterial chromatin. J. Bacteriol. 68 : 464–473. 1954.
7. HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes. Biochem. J. 45 : 353–362, 363–370. 1949.
8. HUEBSCHMAN, C. A method for varying the average number of nuclei in the conidia of *Neuropsora crassa*. Mycologia, 44 : 599–603. 1952.
9. LEMOIGNE, M., DELAPORTE, B., and CROSON, M. Contribution a l'étude botanique et biochimique des bactéries du genre *Bacillus*. Ann. inst. Pasteur, 70 : 224–233. 1944.
10. LOGAN, J. E., MANNELL, W. A., and ROSSITER, R. J. Estimation of nucleic acid in tissue from the nervous system. Biochem. J. 51 : 470–482. 1952.
11. MALMGREN, B. and HEDEN, C. G. Studies of the nucleotide metabolism of bacteria. I. Ultraviolet microspectrography as an aid in the study of the nucleotide content of bacteria. Acta Pathol. Microbiol. Scand. 24 : 417–436. 1947.
12. MIRSKY, A. E. Chemical properties of isolated chromosomes. Cold Spring Harbour Symp. quant. Biol. 12 : 143–146. 1947.
13. MURRAY, R. G. E. Symposium on bacterial cytology 6th Intern. Congr. of Microbiol. Rome pp. 136–138. 1953.
14. PARK, J. T. Uridine-5′-pyrophosphate derivatives. J. Biol. Chem. 194 : 877–904. 1952.
15. PARK, J. T. and JOHNSTON, M. J. Accumulation of labile phosphate in *Staphylococcus aureus* grown in the presence of penicillin. J. Biol. Chem. 179 : 585–592. 1949.
16. POWELL, J. F. Factors affecting the germination of thick suspensions of *B. subtilis* spores in L-alanine solution. J. Gen. Microbiol. 4 : 330–338. 1950.
17. POWELL, J. F. Isolation of dipicolinic acid from spores of *Bacillus megaterium*. Biochem. J. 54 : 210–211. 1953.
18. POWELL, J. F. and STRANGE, R. E. Biochemical changes occurring during the germination of bacterial spores. Biochem. J. 54 : 205–209. 1953.
19. PREUNER, R. Untersuchungen über den Bakterienkern. Arch. Hyg. u. Bakteriol. 134 : 281–294. 1951.
20. PRICE, W. H. Phage formation in *Staphylococcus muscae* cultures. The synthesis of ribonucleic acid, desoxyribonucleic acid and protein in uninfected bacteria. J. Gen. Physiol. 35 : 741–759. 1951.
21. RACKER, E. Enzymatic synthesis and breakdown of desoxyribose phosphate. J. Biol. Chem. 196 : 347–365. 1952.
22. ROBINOW, C. F. A study of the nuclear apparatus of bacteria. Proc. Roy. Soc. (London), B, 130 : 299–324. 1942.
23. ROBINOW, C. F. *Addendum to* The bacterial cell by R. J. Dubos, Harvard Univ. Press, Cambridge, Mass. 1945.
24. ROBINOW, C. F. Observations on the nucleus of resting and germinating spores of *Bacillus megaterium*. J. Bacteriol. 65 : 378–382. 1953.
25. ROBINOW, C. F. Spore structure as revealed by thin sections. J. Bacteriol. 66 : 300–311. 1953.
26. SCHMIDT, G. and THANNHAUSER, S. J. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phospho-proteins in animal tissues. J. Biol. Chem. 161 : 83–89. 1945.
27. SCHNEIDER, W. C. Phosphorus compounds in animal tissues. J. Biol. Chem. 161 : 293–303. 1945.
28. SPENCER, R. E. J. and POWELL, J. F. Flavin-adenine dinucleotide and diaphorase in resting and germinated spores and vegetative cells of *Bacillus subtilis* and *Bacillus megaterium*. Biochem. J. 51 : 239–245. 1952.
29. WEIBULL, C. Characterization of the protoplasmic constituents of *Bacillus megaterium*. J. Bacteriol. 66 : 696–702. 1953.

THE INHIBITING EFFECT OF SOME HETEROCYCLIC AND OTHER ORGANIC COMPOUNDS ON TOBACCO MOSAIC VIRUS[1]

By M. Weintraub[2] and W. G. Kemp[3]

Abstract

A number of heterocyclic and miscellaneous organic compounds have been tested for their effectiveness as virus inhibitors by a half-leaf technique using *Nicotiana glutinosa* and tobacco mosaic virus. Several of these compounds were found to be effective in varying degrees, the main effects being a reduction in the total number of lesions produced, a delay in symptom expression, and a decrease in virus multiplication as indicated by small lesions, on the treated half-leaves. Although nothing is known about the mechanism of inhibition, it can be concluded that to effect inhibition compounds act through the physiology of the host, rather than directly on the virus. The virus content of treated half-leaves has been estimated by measuring their rate of oxygen consumption. These data indicate that the inhibiting compounds affect the metabolism of the host, as reflected in a change in respiration when compared to control half-leaves, and that the symptomless areas on the treated half-leaves do not contain significant concentrations of virus.

Introduction

In continuation of the screening tests previously reported (8), a number of organic compounds were tested for their effectiveness in inhibiting tobacco mosaic on *Nicotiana glutinosa*. The first selection of compounds was, of necessity, rather haphazard. Too little was known about the mechanisms of virus inhibition to provide a clear hypothesis and logical plan of research. As a result, compounds were selected primarily on the basis of their being known to exert some inhibiting action on biological material such as bacteria or fungi.

After one or two compounds had been found to inhibit virus activity, it became possible to choose compounds on a more selective basis.

Methods

The methods used in these experiments were essentially those reported in an earlier publication (8). In brief, the method adopted was a partial *in vivo* one, in which the effects of each compound were tested upon half-leaves of *N. glutinosa* before and after inoculation with the virus. For the former, four left halves and four right halves of leaves were floated in the test compound (in aqueous solution, emulsion, or mechanical mixture), the corresponding halves in distilled water, for 24 hr. The half-leaves were then inoculated with a mixture of purified tobacco mosaic virus and carborundum, and replaced in their respective liquids, until lesions developed and were counted.

For treatment after inoculation, the half-leaves were inoculated, and then floated on distilled water for six hours. Four left halves and four right halves

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were then transferred to the test compound, the corresponding halves remaining in water.

All the experiments were carried out in air-conditioned chambers at a constant temperature of 75° F., and illuminated for 12, or in some cases 18, hours daily, at a constant intensity of approximately 1200 ft-c. In this way, the half-leaves tended to remain in better condition throughout the tests.

All compounds were first tested at the arbitrary concentration of 1 part in 1000 of water, by volume if a liquid and by weight if a solid. If this concentration resulted in injury, the compound was retested at lower concentrations. Similarly, if a particular compound did not inhibit virus activity and did not induce injury at 1 : 1000, it was again tested at a higher concentration, the only exceptions being a few compounds not available in sufficient quantity.

The data were analyzed statistically for significance of differences between treated half-leaves and water controls. In general, Student's 't' test was applied directly, but where total inhibition of lesions was produced in the treated leaves, Kleczkowski's transformation (4) was used. This transformation was $Y = \log(x + c)$, where $x =$ number of lesions and $c =$ constant, usually taken as 1.

Results

In Vivo Experiments

The following miscellaneous compounds were found to be inactive in the inhibition of virus activity, at all concentrations tested:

Methylcarbamic acid (ethyl ester), sodium diethyldithiocarbamate, ferric dimethyldithiocarbamate, zinc dimethyldithiocarbamate, tetramethylthiuram disulphide, tetraethylthiuram disulphide, 4-chloro-3,5-dimethylphenoxyethanol (in liquid and paste form);

Thiophene, 2-thiophenecarboxaldehyde semicarbazone, 2-thiopheneacrylic acid, 2-thiophenesulphonyl chloride, thionaphthene, 3,4-diamino-tetrahydrothiophene-2-valeric acid sulphate, 4-benzamido-3-keto-tetrahydrothiophene, and 4-benzamido-3-keto-Δ2,8-tetrahydrothiophene-2-valeric acid (the last three compounds being intermediate products in the synthesis of biotin);

Furan, 5-nitrofurfural diacetate, 5-nitrofurfural semicarbazone;

5-Nitro-2-furfuraldoxime was severely injurious to the leaves at all concentrations down to 1 : 100,000, at which point no significant reduction was produced. Hydrofuramide also injured the leaves at concentrations of 1 : 1000 and 1 : 5000, but at the latter concentration produced small reductions in some tests, and none in others;

Thionine, benzophenone, hydrobenzamide, hexamethylenetetramine, and heptaldehyde.

Table I shows those heterocyclic derivatives that reduced the number of lesions produced by the virus. Of the 11 thiophene derivatives tested, only three were found to possess inhibitory activity (see Fig. 1), while the other eight, including thiophene itself, had no significant effect in reducing lesion production.

PLATE I

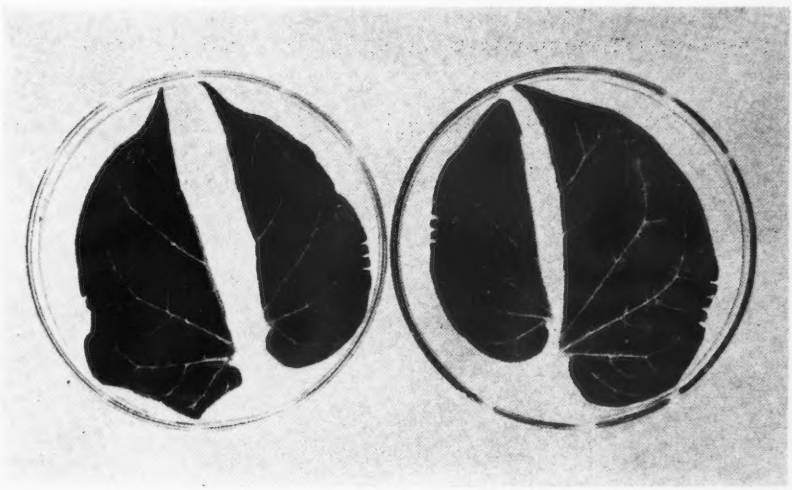

FIG. 1. Inhibition of tobacco mosaic virus lesions on half-leaves of *Nicotiana glutinosa*. Petri dish on left contains two separate half-leaves floating in aqueous 2-thiophene carboxaldehyde thiosemicarbazone (1 : 1000), 48 hr. after inoculation. Note lack of lesions. Petri dish on right contains the opposite half-leaves floating in water, 48 hr. after inoculation. Note plentiful lesions.

TABLE I

EFFECT OF HETEROCYCLIC AND MISCELLANEOUS COMPOUNDS ON PRODUCTION OF LESIONS BY TOBACCO MOSAIC VIRUS ON DETACHED HALF-LEAVES OF *Nicotiana glutinosa*

Compound	Concentration	Mean % reduction	
		Treatment before inoculation	Treatment after inoculation
2-Thiophenecarboxaldehyde thiosemicarbazone	1 : 1000	90.6‡	77.8‡
	1 : 5000	68.7‡	46.7‡
	1 : 10,000	36.9	N.S.R.*
	1 : 100,000	N.S.R.*	N.S.R.
2-Benzoyl thiophene	1 : 1000†	About 100	About 100
	1 : 5000†—48 hr. after inoc.	About 100	About 100
	1 : 5000—72 hr. after inoc.	About 100	About 100
	1 : 10,000—48 hr. after inoc.	About 100	About 100
	1 : 10,000—72 hr. after inoc.	About 100	About 100
	1 : 10,000—80 hr. after inoc.	67.7‡	—
2-Acetyl thiophene	1 : 1000	85.4‡	92.9‡
	1 : 5000	Usually N.S.R.‡	Usually N.S.R.‡
	1 : 10,000	N.S.R.‡	N.S.R.‡
	1 : 100,000	N.S.R.	N.S.R.
2-Furaldehyde	1 : 1000†	100	100
	1 : 5000	N.S.R.‡	N.S.R.‡
2-Furaldoxime	1 : 1000	100	88.4‡
2-Nitrofurane	1 : 1000	—	—
	1 : 5000—48 hr. after inoc.	85.9	97.5
	1 : 5000—72 hr. after inoc.	N.S.R.	N.S.R.
	1 : 10,000	N.S.R.	N.S.R.
Furamide	1 : 500	52.9	N.S.R.
	1 : 1000	N.S.R.	N.S.R.
Acetophenone	1 : 1000	100	100
Veratraldehyde	1 : 1000	44.6‡	N.S.R.‡
Propiophenone	1 : 5000—48 hr. after inoc.	84.9‡	52.7‡
	1 : 5000—72 hr. after inoc.	N.S.R.‡	N.S.R.‡
	1 : 10,000	N.S.R.	N.S.R.

* *N.S.R. = No significant reduction.*

† *Leaves injured by compound.*

‡ *Lesions on the treated half-leaves, where present, very small and indistinct, as compared to the control halves.*

From these results it was reasoned that other heterocyclic compounds, including furan derivatives, might also act as inhibitors. Of those tested four were active, while the five listed above had no effect. Once again, furan itself, like thiophene, did not inhibit lesion production.

It is noteworthy that although 2-thiophenecarboxaldehyde thiosemicarbazone was a very effective inhibitor, the closely related 2-thiophenecarboxaldehyde semicarbazone had no effect whatsoever. Results of this

sort made it difficult to decide how to determine the particular chemical structure required for inhibitory activity. Among the possibilities tested was one wherein an aldehyde or ketone was required, and compounds were selected so that these groups would go into solution slowly. Hexamethylenetetramine, for example, will slowly produce formaldehyde in solution. At the same time, compounds were tested to determine whether aldehydes associated with other than heterocyclic ring structures, or without a ring nucleus at all, were also effective as inhibitors.

As a result of these tests, three more inhibitory compounds were detected: acetophenone, veratraldehyde, and propiophenone (Table I). However, of this group, benzophenone, hexamethylenetetramine, hydrobenzamide, and heptaldehyde were inactive.

It will be noted that, in general, treatment before inoculation resulted in greater reduction of lesion numbers than did treatment after inoculation. In several instances, as with 2-thiophenecarboxaldehyde thiosemicarbazone, furamide, and veratraldehyde, the dilution end-point for inhibitory activity was reached at a greater concentration for treatment after, than for treatment before, inoculation. It was difficult, occasionally, to determine the exact dilution end-point for inhibitory activity, as, for example, with 2-acetyl thiophene. In some tests a concentration of 2×10^{-4} produced slight but significant reduction in lesion numbers, while in other tests it produced no significant reduction. This was probably due to the variation in leaf materials, and the resulting difference in the total amount of the compound absorbed by the half-leaves.

Nothing is known, of course, about the final concentration of inhibitory compounds at the sites of virus infection, nor is the active concentration of the compounds in the liquid medium known. 2-Thiophenecarboxaldehyde thiosemicarbazone, for example, is relatively insoluble in water at room temperature. In one experiment, equal amounts of this compound were prepared and tested in three ways: (1) stirred in water at room temperature; (2) totally dissolved in boiling water; crystals formed on cooling were left in mixture; (3) dissolved in boiling water, filtered after cooling. It was found that for treatment before inoculation, boiling the compound without subsequent filtering produced slightly greater inhibition (about 10%) than did the other two methods. For treatment after inoculation, boiling, with or without filtration, produced from two to three times the inhibition that only stirring at room temperature did. It is evident, then, that with some of the heterocyclic compounds with low solubility, the active concentration is unknown, unless the amount in solution is analyzed.

To a lesser or greater extent, the inhibitory action of all the compounds was diminished with increase of time after inoculation. Thus, both 2-nitrofurane and veratraldehyde produced substantial reductions in the number of lesions, if lesions were counted 48 hr. after inoculation. However, a recount of the lesions 24 hr. later showed no significant difference between the treated and control halves. The reduction in lesion numbers by 2-benzoyl thiophene

was more constant in this respect. Table I shows that at concentrations of 2×10^{-4} and 10^{-4}, lesion counts made at 48 and 72 hr. after inoculation showed total inhibition of virus lesions on the treated halves. It was only after 80 hr. that the amount of reduction in lesion numbers diminished, as lesions appeared on the treated halves. Unfortunately, it was not possible to count the lesions after this time, because of the deterioration of the half-leaves in the Petri dishes.

Lesion Size

It will be noted in Table I that in several cases, marked with the superscript "‡", treatment with the various compounds had a further effect on the size and appearance of the lesions. This was true even in several instances where the dilution end-point of the compound's inhibiting activity had been reached and no significant reduction of lesion numbers was produced. The lesions produced on the treated half-leaves in these instances were regularly smaller and considerably less necrotic than those on the control halves. Quite frequently it was necessary to resort to a strong light shining through such treated half-leaves to count the tiny brown-, gray-, or green-colored lesions.

In several experiments, however, the lesions on the treated leaves were well enough formed so that their size could be measured. This was done with a binocular microscope containing a calibrated ocular, the measurements being made across the largest diameter of the lesions, both on treated half-leaves and the corresponding controls. In one such set of measurements 80 lesions on the treated half-leaves had a mean diameter of $0.91 \pm .03$ mm., while those on the control halves had a mean diameter of $2.16 \pm .04$ mm.

Environmental Effects

During the winter of 1952–53, beginning in December, and continuing into February, heterocyclic compounds that until that time had been found to inhibit lesion production became ineffective. The number of lesions on the treated half-leaves was, at this time, not significantly different from that on the control halves. After a number of repeated tests, it was thought that the poor growing conditions in the greenhouses might be responsible. At that time of year, of course, light intensity was low, and its duration relatively short, while daily temperatures in the greenhouses fluctuated considerably.

A number of plants were therefore removed to the air-conditioned chambers to increase light intensity and duration, and to keep the temperature constant. After a week to 10 days, leaves from these plants were tested with several of the known inhibitors. Since inhibition again became operative under these conditions, it is evident that the physiological state of the host, as influenced by environment, was responsible for the temporary failure of inhibitory activity.

After it had been established that the condition of the host played a part in the inhibitory activity of the several compounds, experiments were subsequently planned so that whenever new compounds were to be tested, one of

the known inhibitors was always included in the test. In this way, lack of inhibition with a new compound could more certainly be attributed to the compound itself, rather than to the physiological state of the plant. The same phenomenon occurred in the winter months of 1953–54.

In Vitro Experiments

In order to obtain additional evidence as to whether the inhibitors were acting directly on the virus, or through the host, a number of *in vitro* experiments were made. The compounds, at inhibiting concentrations, were mixed with purified tobacco mosaic virus solution, in equal portions. These mixtures were applied to half-leaves of *N. glutinosa* at 0, 6, and 24 hr. after mixing. The opposite halves of these leaves were inoculated with an equal quantity of virus solution diluted with water to the same concentration as the treated virus. 2-Thiophenecarboxaldehyde thiosemicarbazone, 2-benzoyl thiophene, 2-acetyl thiophene, 2-furaldehyde, and 2-furaldoxime were tested in this way. No significant reduction in the number of lesions was produced by any of the treated virus solutions.

Tests with Intact Plant Material

A limited number of experiments were made with potted plants, rooted cuttings, and buds. Potted *Datura stramonium*, *N. glutinosa*, and *N. tabacum* were sprayed, or the soil drenched with the several compounds, before, simultaneously with, and after inoculation with tobacco mosaic virus. The roots of *Dianthus barbatus* cuttings, previously rooted in vermiculite, were soaked in the several compounds, then potted and tested for inhibition with carnation mosaic virus. Cherry buds infected with cherry yellows virus were soaked in the compounds, then grafted to healthy cherry trees. Appropriate controls were set up in each test. None of these trials resulted in inhibition of the virus involved, although some delay in symptom expression was observed in *D. stramonium* inoculated with tobacco mosaic virus, and in a few of the cherry buds.

Virus Content in the Treated Leaves

One of the disadvantages of the screening technique used here is that the actual virus concentration within the treated half-leaves is unknown. It is possible that the compounds appearing to affect the virus infection may not be delaying or inhibiting multiplication of the virus at all, but may only be interfering with necrotic processes, that is, with symptom expression. If this is the case, then two possibilities suggest themselves, the first that the virus is present and has multiplied to the same extent in the treated leaves as it has in the controls, and is still localized, despite the lack or decrease of necrosis. The second possibility is that the virus multiplies in the treated half-leaf unimpeded by the necrotic processes that usually localize viruses in hypersensitive hosts. Under these conditions, the result would be systemic infection of the treated half-leaf.

Measuring the virus content of the half-leaves by infectivity tests was made unsatisfactory by the presence of virus left on the surface of all the half-leaves from the inoculation. Some tests soon disclosed the fact that it was not possible to rid the surface of a great part of this virus in less than 20 hr. of constant washing. This made direct assay of the virus content of the half-leaves unsatisfactory, so that an indirect method was used.

Recent work at this laboratory (9) has shown that the respiration of *N. glutinosa* leaves inoculated with tobacco mosaic virus is raised three to four times that of uninoculated leaves or of leaves rubbed with water and carborundum. Furthermore, although only those portions of the inoculated leaves that contain lesions, and the tissues immediately surrounding the lesions, show this rise, the respiration of leaf disks from such areas begins to increase until it is twice that of uninoculated controls, as long as six hours before the first sign of lesions can be detected. It is evident therefore that this physiological disturbance is correlated primarily with multiplication of the virus at local sites of infection rather than with the necrotic process as such. It can thus be used as a symptomatic guide to the question of whether the treated, but symptomless, leaves contain the same concentration of localized virus as do the control leaves.

To this purpose, a test was set up as described above for treatment before inoculation, the half-leaves for treatment being placed in Petri dishes containing 2-thiophenecarboxaldehyde thiosemicarbazone, the control halves in water, and all inoculated 24 hr. later. After lesions had developed over the whole area of the water-control halves, disks 11 mm. in diameter were cut from approximately identical positions of the opposite half-leaves of both the treated and control. In addition to these, disks were taken from half-leaves that had not been inoculated at all, but had been floated for the same length of time in Petri dishes containing water, and in dishes containing the virus inhibitor. Four such disks were placed in each Warburg vessel, and the respiration, as oxygen uptake, was measured over a three hour period.

The half-leaves from which the disks were taken were kept, and within another 24 hr., lesions began to appear on the treated, inoculated half-leaves, although these lesions were smaller and considerably less necrotic than those on the inoculated control halves. At this time, disks were again cut from the treated, inoculated half-leaves from areas that now contained lesions and also from the same leaves from areas that did not contain lesions. Similar disks were cut from the opposite, inoculated control half-leaves, from approximately the same areas as on the treated halves. At the same time, disks were once more taken from the uninoculated half-leaves floating on the inhibitor and on water. The respiration of these disks was also measured over a three hour period.

Fig. 2 shows the average results of four such determinations. Although oxygen consumption is shown here in terms of fresh weight, essentially the same results were obtained when calculations were based on dry weights. It is evident that, as mentioned above, the disks from inoculated control leaves

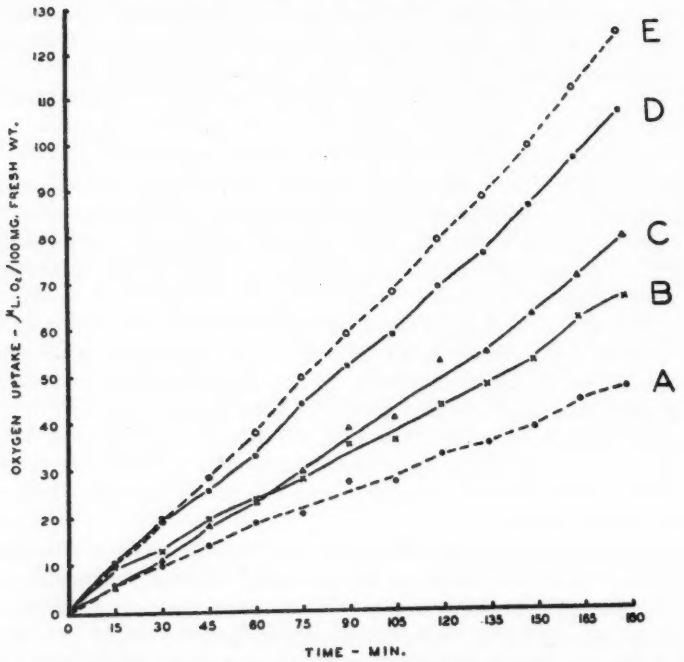

FIG. 2. Oxygen uptake of disks from *Nicotiana glutinosa* half-leaves. Control disks were cut from half-leaves floated for 42 or 65 hr. in water before respiration measurements. Treated disks were cut from half-leaves floated for 42 or 65 hr. in 2-thiophene carboxaldehyde thiosemicarbazone before respiration measurements. *A*. Control disks from uninoculated leaves, lesions absent. *B*. Treated disks from uninoculated leaves, lesions absent. *C*. Treated disks from inoculated leaves, lesions absent. *D*. Treated disks from inoculated leaves; lesions present. *E*. Control disks from inoculated leaves; lesions present.

respired almost three times as rapidly as did disks from uninoculated control leaves. Disks from half-leaves floated in the thiophene derivative, either uninoculated or inoculated but without lesions because of the treatment, respired slightly more rapidly than did the uninoculated controls. (The differences between the uninoculated water controls and the two treated sets, uninoculated and inoculated but symptomless, were significant by Student's 't' test, with $P = .02$, and $> .001$ respectively). On the other hand, there was no significant difference between the respiration of the disks from the uninoculated half-leaves floated in the inhibitor, and that of the disks from inoculated but symptomless half-leaves floated in the inhibitor.

The disks cut from lesioned areas on the inoculated, treated half-leaves respired at a rate comparable to that of lesioned disks from inoculated control half-leaves, and, at a significantly greater rate than either inoculated, treated half-leaves without lesions, or uninoculated half-leaves treated either in the inhibitor or in water.

Thus, in summary, these results indicate that the inhibitor had a basic effect on the host of raising the respiration slightly. Furthermore the respiration of disks from inoculated areas without lesions, whether taken from the treated leaves before or after any symptoms appear, was not significantly different from that of uninoculated disks treated with the inhibitor. Only disks cut from those areas of the treated leaves that actually contained lesions at the end of the test respired at a high rate comparable to that of the lesioned areas cut from the inoculated water controls.

The possibility that virus was present in the treated leaves as a systemic infection, rather than in localized areas without necrosis, is an unlikely one. The possibility was tested, however, by inoculating one-half only of whole leaves of *N. glutinosa*, and floating these on inhibiting compounds, and others on water. Each leaf was split partially along the midrib, to allow better absorption of the compound. After varying intervals of time, up to five days after inoculation (by which time the leaves had deteriorated badly), the uninoculated half of each leaf was carefully cut away, and tested for presence of virus. In all cases, the results were negative, indicating that if virus was present in systemic form in the inoculated halves of the treated leaves, it did not, at least within five days after inoculation, pass over into the uninoculated half of the leaf.

Discussion

There have been several recent reports on the effect of heterocyclic compounds both as fungus and virus inhibitors, as well as on their other biological activity. Davis, Lo, and Dimond (1) concluded that only five-membered heterocyclic rings condensed with two benzene rings are active as chemotherapeutants for fusarium wilt of tomato. Emerson (2), working with dormant ascospores of *Neurospora crassa*, postulated that although a heterocyclic ring is not necessary, inclusion of a furan ring increases the power to activate spores by certain unsaturated ring compounds, nearly all of which are aldehydes.

Minton, Officer, and Thompson (5) and Thompson *et al.* (7) believed that the thiosemicarbazone group is essential for activity against vaccinia infection in the mouse. They postulated further (5) that the thiosemicarbazones with antivaccinal activity do not inhibit multiplication of the virus per se, but possibly permit the infected cells to carry on their functions through minimization of physiological injury. Schlegel and Rawlins (6), using a heat-ultraspectrophotometric method of virus assay, reported benzaldehyde thiosemicarbazone as an inhibitor of tobacco mosaic virus production in an artificial light environment.

Unfortunately, these references do not help to provide a clue to the mechanism of inhibition by the various heterocyclic and miscellaneous compounds described here. The only firm conclusion that can be reached in this respect is that certain heterocyclic derivatives are very effective in delaying and decreasing virus multiplication. Why some heterocycles are effective and others, apparently closely related in structure, are not is not as yet explainable.

However, heterocyclic compounds are apparently not essential for this activity, since some aromatic aldehydes and ketones are active in the same way as the heterocycles. Again, it is difficult to determine why some of the latter act as inhibitors, while others do not, although there are indications that the aldehyde structure alone, without a ring nucleus, is not sufficient for inhibition.

The primary effect of the inhibiting compounds is to delay symptom expression. However, this is not the only effect, since half-leaves treated with some of the compounds showed considerable reduction of lesion numbers even 80 hr. after inoculation as compared with the control halves at that time. In addition to the over-all reduction in numbers of lesions, the character of the lesions on the treated half-leaves indicated that the virus was not as active, and was multiplying less rapidly than on the controls, since these lesions were considerably smaller and less necrotic. Nothing can be said definitely as to whether the inhibitors are more effective in preventing virus establishment or virus multiplication. It is probable that both are operative, since treatment for 24 hr. before inoculation often resulted in somewhat greater reduction of lesion numbers, while at the same time, the small size of the lesions on the treated half-leaves indicated a reduction in the invasiveness, and hence, multiplication of virus already established.

It can be concluded with some certainty that the inhibitors do not act directly on the virus but, rather, through the physiology of the host to resist infection and virus multiplication. The respiration data (Fig. 2) show that the compounds do exert an over-all metabolic effect on the treated leaves, in the case of 2-thiophene carboxaldehyde thiosemicarbazone, reflected in a rise in respiration. Furthermore, it has been demonstrated that the environment determines to a large extent whether the inhibitors are active or not. It is probable that environmental factors such as light and temperature will effect changes in the physiological state of the host that make it more or less amenable to whatever influence the inhibitors exert in order to resist virus infection. In addition to this evidence, the lack of inhibition that results from *in vitro* contact between the virus and the several inhibitors lends further corroboration. that the effect is not on the virus, but on the host.

Some of the theoretical objections to the technique used for screening compounds in these studies have, in part, been removed. The respiration measurements leave little doubt that in those areas of the treated leaves that do not show lesions, there is not simply a suppression of symptoms, but there is either no virus, or there is so little virus present that it is not reflected in the expected rise in respiration. As mentioned previously, it is not necessary to see the lesions to get this rise in respiration, since oxygen uptake begins to increase significantly some six hours before lesions are detectable. It would be expected, therefore, that if actively multiplying virus were present in the symptomless areas of the treated half-leaves, disks from these areas would respire at an increased rate. The lack of such an increase would indicate the lack of any significant concentration of the virus in these areas.

A confirming line of evidence lends weight to the conclusion that this screening technique does not uncover compounds that merely suppress

expression of symptoms, but those that actually affect the virus infection. This confirming evidence is a report by Fulton (3) that zinc chloride and zinc sulphate, found earlier (8) to be effective against tobacco mosaic virus and carnation mosaic virus, using the methods here described, inactivated Type 2 virus infected Robinson strawberry plants in pots, by application of the solutions every four days for 40 days.

A more practical objection to the screening technique using half-leaves of *N. glutinosa* was made by Yarwood (10). He confirmed the report (8) that zinc decreased the number of virus lesions on *N. glutinosa*, but found that the number and size of lesions produced by tobacco mosaic virus on bean leaves was increased with zinc treatment. He questions, therefore, the general application of this particular screening method. Unfortunately, there are limitations to the number of tests that can be made, dictated by considerations of time and space. Even if, in future, bean leaves were included in all tests, it would probably be found that a compound inhibiting tobacco mosaic virus in both *N. glutinosa* and bean would be ineffective against the same virus in some other host, or against another virus in the same hosts. The limitations mentioned, as well as Yarwood's objection, are applicable to all screening techniques for chemotherapeutants so far reported, although a search for a panacea for virus infection is undoubtedly commendable.

Acknowledgments

We are indebted to Mr. J. B. Robinson, Dr. R. S. Willison, and Mr. M. Zoccoli, of this laboratory, and to Prof. G. F. Wright, Chemistry Department, University of Toronto, for assistance in various phases of this work. Thanks are also due to the following, for their samples of the various test compounds: Allied Chemical and Dye Corp., Carbide and Carbon Chemicals Co., CIBA Ltd., Merck and Co., Monsanto Chemical Co., and the Chemistry Department, University of Toronto.

References

1. Davis, D., Chien-Pen Lo, and Dimond, A. E. Chemotherapeutic activity of unsubstituted heterocyclic compounds. Phytopathology, 44 : 680-683. 1954.
2. Emerson, M. R. Some physiological characteristics of ascospore activation in *Neurospora crassa*. Plant Physiol. 29 : 418-428. 1954.
3. Fulton, R. H. Studies on inactivation of the strawberry Type 2 virus *in vivo*. Phytopathology, 44 : 489. 1954. Abstract.
4. Kleczkowski, A. The transformation of local lesion counts for statistical analysis. Ann. Appl. Biol. 36 : 139-152. 1949.
5. Minton, S. A., Jr., Officer, J. E., and Thompson, R. L. Effect of thiosemicarbazones and dichlorophenoxy thiouracil on multiplication of a recently isolated strain of variola-vaccinia virus in the brain of the mouse. J. Immunol. 70 : 222-228. 1953.
6. Schlegel, D. E. and Rawlins, T. E. A screening test of the effect of organic compounds on production of tobacco mosaic virus. J. Bacteriol. 67 : 103-109. 1954.
7. Thompson, R. L., Minton, S. A., Jr., Officer, J. E., and Hitchings, G. H. Effect of heterocyclic and other thiosemicarbazones on vaccinia infection in the mouse. J. Immunol. 70 : 229-234. 1953.
8. Weintraub, M., Gilpatrick, J. D., and Willison, R. S. The effect of certain water-soluble compounds on virus infection. Phytopathology, 42 : 417-419. 1952.
9. Weintraub, M. and Kemp, W. G. Unpublished data.
10. Yarwood, C. E. Zinc increases susceptibility of bean leaves to tobacco mosaic virus. Phytopathology, 44 : 230-233. 1954.

A STUDY OF CERTAIN FACTORS INFLUENCING SPORULATION OF *SACCHAROMYCES CEREVISIAE*[1]

By J. J. Miller[2], James Calvin[3], and J. H. Tremaine[4]

Abstract

A decline in sporulation noted with cells from cultures aged 7–30 days was not due to a decrease in the proportion of viable cells. The optimum cell population density for sporulation in acetate solution was two to four millions per milliliter and seemed unaffected by acetate concentration. Cells from three presporulation media that allowed moderate growth sporulated poorly in acetate solution. Seven assimilable sugars stimulated sporulation at low concentration in water agar, while three non-assimilable sugars and mannitol did not. Glucose-1-phosphate, fructose-1,6-diphosphate, and sodium aspartate were stimulating. The following were non-stimulating: sodium formate, sodium propionate, propionic acid, sodium oxalate, oxalic acid, sodium citrate, citric acid, aminoacetic acid, glutaric acid, indole-3-acetic acid, acetaldehyde, ethyl alcohol, histamine, glucosamine. Differences are pointed out between the chemical conditions favoring growth and sporulation.

Introduction

Stantial (15, 16) found dilute solutions of certain sugars or of acetates, or both, to be very effective in stimulating sporulation of yeast cells, and this was confirmed by Elder (8, 9). Adams (1) was able to simplify Stantial's procedure by preparing presporulation and sporulation media in the solid form, and later (2), in a study with 43 yeast strains, showed that an acetate–glucose agar medium was on the whole superior to Gorodkowa's agar and water agar as a sporulation medium. Adams and Miller (3) found that carbon dioxide inhibited sporulation of *Saccharomyces cerevisiae* on acetate–glucose agar, while oxygen was stimulating. In a comparison of the growth factor requirements for growth and sporulation, Tremaine and Miller (17) concluded that the latter process was somewhat more exacting. This paper describes the results obtained in a related study of other factors involved in the change of yeast cells from the vegetative to the sporulating condition.

Materials and Methods

The following cultures, isolated from commercial packaged yeasts, were employed: L (Lallemand); B (Best); FJ, F48, F54A, F54B (Fleischmann). Stock cultures were maintained in the refrigerator on agar slants or butts covered with mineral oil as recommended by Henry (11). Vegetative cells for sporulation experiments were obtained, unless otherwise stated, by growing the yeast for two days on a presporulation medium. Since these media were varied, they will be described in connection with the individual experiments. Sporulation cultures were prepared by spreading yeast cells over the

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Contribution No. 66 from the Department of Biology, Hamilton College, McMaster University, Hamilton, Ontario. The work was supported by a grant from the National Research Council of Canada.
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[3,4] *At the time, graduate students.*

surface of slants of water agar containing a given compound, or by adding the cells to solutions dispensed in shell vials (15 × 45 mm.). The volume of the latter cultures was 0.8 ml., which gave a depth of approximately 0.5 mm. Adams (1) has shown that sporulation is diminished in deeper cultures. The percentage ascus formation in sporulation cultures was estimated by examining at random 200 cells from each.

Experimental

Sporulation of Cells from Cultures of Varying Ages

Cells from young sporulation cultures have been shown (4, 9) to give highest sporulation yields, and it has been pointed out (1) that this could result from an increase in the number of dead cells in older cultures.

TABLE I

SPORULATION OF CELLS FROM CULTURES OF VARYING AGES AFTER TWO WEEKS ON ACETATE–GLUCOSE AGAR

Age of culture, days	Yield of asci, %				
	Experiment 1			Experiment 2	
	Strain FJ	Strain L	Strain B	Strain FJ	Strain F48
1	28.0	24.0	17.8	33.7	30.0
2	23.3	24.3	26.5	36.5	34.5
4	27.5	27.7	28.2	26.2	33.8
7				18.8	20.5
8	23.0	20.0	24.0		
14				14.5	15.7
16	18.8	14.8	14.8		
30	12.3	12.8	6.0	10.7	7.0

Three yeast strains were inoculated on slants of nutrient agar (Difco) containing 5% glucose, and transferred after periods of one to 30 days to slants of acetate–glucose agar (1). The results (Table I) show that cells from seven- or eight-day cultures formed fewer asci, and that cells from 14-, 16-, and 30-day cultures gave still lower yields, than did cells from the one-, two-, and four-day cultures. To determine whether this result was due to an increase in the number of dead cells in the older cultures, suspensions in distilled water were prepared from the 7-, 14-, and 30-day cultures used in the second experiment. One-milliliter volumes of the suspensions, adjusted to cell population densities of 100 per ml., were plated with glucose nutrient agar, and the results are shown in Table II. No decline in viability was evident with either strain after 14 days, nor after 30 days with FJ, although F48 appeared somewhat less viable.

It seems clear that aging did not cause low ascus yields by increasing the number of dead cells, and this indicates that the effect is rather due to physiological changes occurring in the senescent cultures. Of interest here is the

TABLE II

VIABILITY OF TWO YEAST STRAINS ON GLUCOSE NUTRIENT AGAR

Strain	Percentage of viable cells after:			
	0 days*	7 days	14 days	30 days
FJ	95.8	90.0	92.0	89.3
F48	91.6	96.0	93.7	80.3

** The figures in this column represent the proportion of viable cells in the inoculum, which was obtained from a two-day-old culture on glucose nutrient agar.*

work of Eaton and Klein (7) which suggests that different oxidative patterns may occur in yeast cells depending on the stage in the growth cycle at which they are harvested.

Effect of Cell Population Density and Acetate Concentration on Sporulation

Cells of the FJ strain harvested from 48-hr. cultures on 5% glucose nutrient agar + 0.1% yeast extract (Difco) were placed in 0.1% sodium acetate solution in vials at population densities of 40, 20, 10, 5, 2, 1, and 0.5 million cells per ml. The yields of asci after seven days at 27° C. are shown in Table III. Yields were reduced when the cell population density was above 5 million per ml. or below 1 million per ml.

TABLE III

EFFECT OF CELL POPULATION DENSITY ON SPORULATION IN 0.1% SODIUM ACETATE SOLUTION

Millions of cells per ml.	Yield of asci after 7 days, %
40	0.2*
20	0.3
10	3.6
5	16.4
2	12.4
1	15.9
0.5	5.4

** Each figure based on an examination of 1000 cells; 200 from each of five vials.*

The cell population density was then kept constant at 1 million per ml. while the acetate concentration was varied from 0 to 1.0% (Table IV). Highest yields were observed at 0.1, 0.15, and 0.2%.

The sporulation of cell populations of varying densities was also compared in different concentrations of acetate. Cells from slants of 5% glucose nutrient agar + 1% yeast extract were placed in solutions of sodium acetate

TABLE IV

EFFECT OF SODIUM ACETATE CONCENTRATION ON SPORULATION AT A CELL POPULATION DENSITY OF ONE MILLION PER MILLILITER

Acetate concentration, %	Yield of asci after 7 days, %
1.0	7.6*
0.6	9.7
0.3	13.8
0.2	19.6
0.15	20.4
0.1	23.1
0.05	11.5
0	1.0

* *Each figure based on an examination of 1000 cells; 200 from each of five vials.*

of the following concentrations: 3.0, 1.0, 0.3, 0.1, 0.03, 0.01%. Cell population densities employed were: 16, 8, 4, 2, 1, 0.5, 0.25, 0.13, and 0.063 million per ml. The results of this set of experiments are summarized in Fig. 1, omitting the 3.0% concentration since no spores were found in it. A cell population density of 4 million per ml. seemed optimum for ascospore formation at all acetate concentrations except 0.01 and 1.0%. In the former instance the optimum cell population density was 2 million per ml. and in the latter very few spores formed at any cell population density. The highest yields were observed in the 0.1% acetate solution at cell population densities of 2 and 4 million per ml. With 0.5, 0.1, 8, and 16 million per ml., higher yields were obtained with 0.01% acetate than with 0.1%. Sporulation in 0.03% acetate was less than in either 0.1 or 0.01%. This may be due to the fact that each acetate concentration was studied as an individual experiment, and sporulation during the work with 0.03% acetate may have been depressed by some factor, perhaps a change in the relative abundance of certain genetic types in the stock culture.

The frequency of four-spored asci, shown in Fig. 2, followed in general the same trends as total sporulation. However, with 0.1% acetate the optimum for four-spored asci was 2 million cells per ml., while the optimum for total asci was 4 million per ml. Also, at a cell population density of 4 million per ml. the highest yields of four-spored asci occurred in 0.01% acetate, although total sporulation was highest in 0.1%. It has previously been shown (17) that the proportion of four-spored to total asci may be altered by modifying the presporulation medium.

Stantial (15, 16) concluded that the optimum cell population density varied with the concentration of active substance in the sporulation medium. The foregoing results do not support this view but indicate that, with acetate at least, the concentration does not much affect the optimum cell population density.

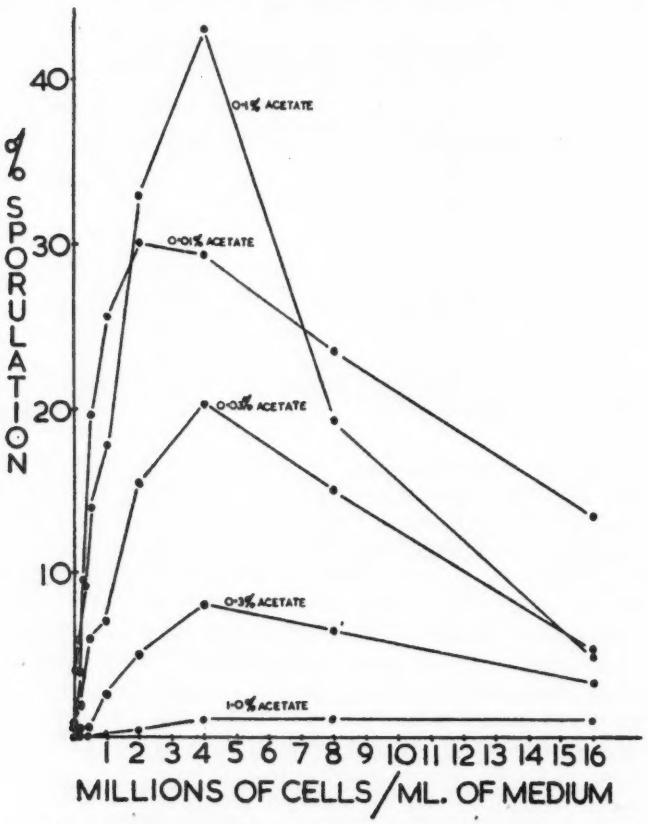

FIG. 1. Influence of cell population density on yields of asci in vials containing five concentrations of sodium acetate.

Sporulation of Cells Cultured on Several Presporulation Media

Cells multiplied in a mineral salts + glucose medium have given yields in acetate–glucose solution less than half as great as cells from the same medium enriched with wort (16). Similarly, yields in 0.1% sodium acetate with cells from tomato juice agar or glucose nutrient agar have been found comparable to those obtained with two chemically-defined media when necessary vitamins were included in the latter (17).

Cells of the FJ strain were grown for two days on the 12 presporulation media listed in Table V, and then used to prepare sporulation cultures with a cell population density of 1 million per ml. in five replicate vials. Cells from tomato juice agar gave the highest yield. No spores were found in cells from Czapek solution agar unless it had been amended with yeast extract or peptone, but cells from the other synthetic medium (Lodder's) gave a low yield. Possibly this is because the nitrogen source in Czapek solution agar

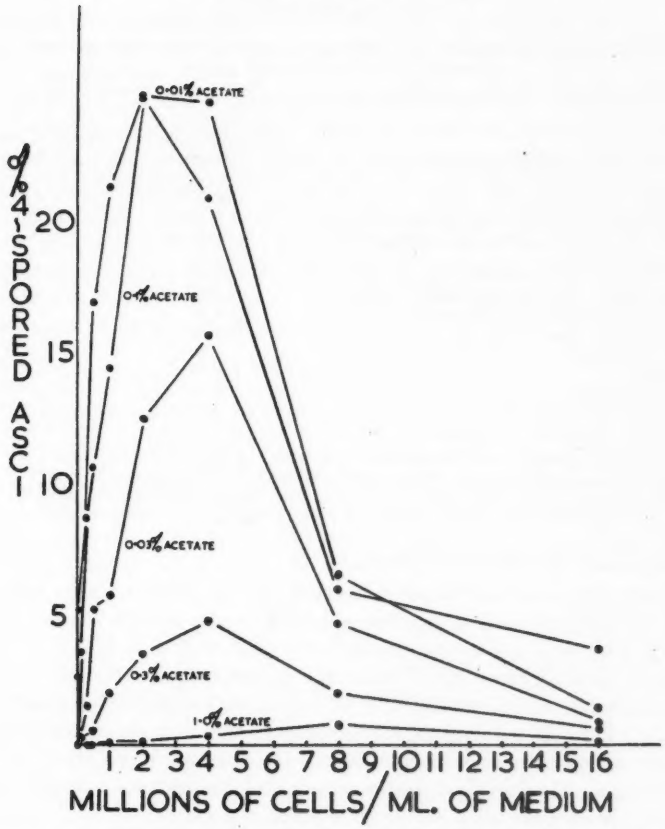

FIG. 2. Influence of cell population density on yields of four-spored asci in vials containing five concentrations of sodium acetate.

is sodium nitrate, whereas in Lodder's medium it is ammonium sulphate. Likewise, glucose nutrient agar is evidently somewhat deficient in factors needed for optimum sporulation. Growth, however, was not appreciably less on glucose nutrient agar than on tomato juice agar or on wort agar. These results support the view (17) that the nutritional requirements for sporulation are more exacting than for growth.

Potato dextrose agar supported growth and sporulation less effectively than either tomato juice agar or wort agar. Elder (9) found growth to occur more readily in carrot extract than in potato extract and, although no ascospores developed in potato extract, they became fairly abundant (14–28%) in carrot extract. Presumably potatoes are deficient in a factor (or factors) that is (or are) more important for sporulation than for vegetative growth.

TABLE V

ASCOSPORE FORMATION IN 0.1% SODIUM ACETATE SOLUTION BY CELLS FROM 12 PRESPORULATION MEDIA

Presporulation medium	Growth on presporulation medium	Yield of asci after 7 days, %
5% Glucose nutrient agar	+++*	16.2†
5% Glucose nutrient agar + 0.1% yeast extract	+++	25.8
5% Glucose nutrient agar + 1% yeast extract	+++	34.3
Czapek solution agar	+	0
Czapek solution agar + 0.1% yeast extract	++	3.7
Czapek solution agar + 1.0% yeast extract	+++	35.2
Czapek solution agar + 0.1% peptone	++	2.1
Czapek solution agar + 1% peptone	+++	22.7
Lodder's medium‡	+	6.9
Wort agar	+++	31.2
Tomato juice agar	+++	37.5
Potato dextrose agar	++	9.4

* + = *slight growth;* ++ = *moderate growth;* +++ = *abundant growth.*
† *Each figure based on an examination of 1000 cells; 200 from each of five vials.*
‡ *2% glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.05% $MgSO_4$, and 2% agar in distilled water. The other five media, yeast extract, and peptone were as supplied by the Difco Corporation.*

Stimulation of Sporulation by Sugars

Glucose stimulates ascospore formation, and this is illustrated by the data shown in Table VI which compare the sporulation of cells from 5% glucose nutrient agar on slants containing 0, 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 10% glucose. With all three yeast strains the optimum concentration was 0.05%. Acetate–glucose agar, containing 0.04% glucose and 0.14% sodium acetate, was much more stimulating than glucose, and this is in accord with Stantial's (16) conclusion that acetate and glucose together are more stimulating than either alone. However, in the experience of the writers acetate + glucose has not proved more stimulating than acetate alone.

TABLE VI

SPORULATION OF THREE STRAINS ON WATER AGAR CONTAINING SEVEN CONCENTRATIONS OF GLUCOSE

Glucose concentration, %	Yield of asci after 14 days, %		
	Strain FJ	Strain L	Strain B
10.0	0.1	0.3	0.5
5.0	0	0	0
1.0	1.1	0.7	0.5
0.5	3.5	0.5	1.2
0.1	7.2	4.8	7.5
0.05	10.3	13.5	11.2
0.01	9.7	11.0	8.7
0	3.1*	2.3	3.2
Acetate–glucose agar†	33.5	34.0	38.2

* *Each figure based on an examination of 600 cells; 200 from each of three tubes.*
† *Formula as prepared by Adams (1).*

In two further experiments cells of strain FJ from the same presporulation medium were spread on slants of water agar containing 10 sugars and mannitol in the concentrations found most stimulating in the foregoing experiment (0.05 and 0.01%). The results are shown in Table VII. There is evidence of stimulation by glucose, fructose, mannose, galactose, maltose, sucrose, and raffinose. Lactose, melibiose, arabinose, and mannitol had no definite effect at the concentrations employed. Since the latter four substances are not assimilated by this strain of yeast, it would seem that a given sugar must be metabolized in order to cause ascospore formation. The fact that raffinose was the least stimulating of the first group is presumably because only one-third of the raffinose molecule is assimilated by this strain. Fructose and mannose were more stimulating than glucose.

TABLE VII

SPORULATION OF STRAIN FJ ON SLANTS OF WATER AGAR CONTAINING 10 SUGARS, MANNITOL, AND ACETATE

Experiment I			Experiment II		
Compound	% compound	Yield of asci, %	Compound	% compound	Yield of asci, %
Glucose	0.01	3.7*	Glucose	0.01	3.0
	0.05	16.5		0.05	10.2
Galactose	0.01	3.5	Fructose	0.01	10.5
	0.05	15.1		0.05	23.3
Maltose	0.01	1.3	Mannose	0.01	11.2
	0.05	14.8		0.05	23.4
Melibiose	0.01	0.8	Sucrose	0.01	3.2
	0.05	1.5		0.05	9.5
Raffinose	0.01	0.9	Lactose	0.01	2.4
	0.05	7.4		0.05	1.7
Arabinose	0.01	1.7	Sodium acetate	0.1	26.3
	0.05	0.3	Water agar only		0.5
Mannitol	0.01	2.0			
	0.05	0.9			
Water agar only		0.9			

* *Each figure based on an examination of 1000 cells; 200 from each of five tubes.*

These results are in agreement with Stantial's results, but whereas she found sucrose and maltose to be more stimulating than glucose, in the present work all three sugars were similar. It is unexpected that fructose and mannose should be more stimulating than glucose. In the following experiment they are again found to give higher yields than glucose. (See Fig. 3.)

Stimulation of Sporulation by Two Hexose Phosphates

Others (10, 5) have observed increased perithecial formation in the presence of hexose phosphates.

A preliminary experiment indicated that both glucose-1-phosphate and fructose-1,6-diphosphate stimulated sporulation at a concentration of 0.05% in distilled water. Their effect on sporulation was then compared over a

range of concentrations in water agar slants, inoculated with cells from glucose nutrient agar + 1% yeast extract (Fig. 3). With fructose-1,6-diphosphate the optimum concentration for sporulation was 0.19%, but with glucose-1-phosphate no decline was evident at the higher concentrations. Glucose, fructose, and mannose were included in the experiment for comparison. The optimum concentration with fructose was 0.01%, and with the other two, 0.05%.

In another experiment glucose-1-phosphate was added to water agar in nine concentrations ranging from 0.0072% to 2.0%. The results are shown in Fig. 4, and for comparison values are included that were obtained in experiments with glucose and sodium acetate done concurrently. With glucose-1-phosphate yields were high at all concentrations above 0.072%, and again no decline was found at the higher concentrations. Glucose showed an optimum at 0.01% although in the previous experiment its optimum was 0.05%. With sodium acetate high yields were obtained at 0.05, 0.1, and 0.5% and definite declines occurred above and below these concentrations. The three compounds thus differ in respect of the range of concentrations that favor high yields.

Stantial (16) has shown that phosphate alone is not effective in stimulating sporulation, and that the addition of potassium dihydrogen phosphate to a sporulation medium containing sugar or acetate has little influence on ascus yields. This has been confirmed by Elder (9), and by the writers.

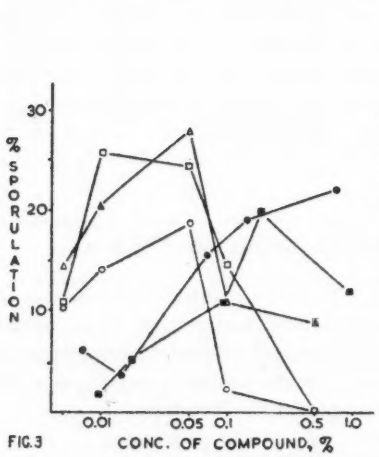

FIG. 3

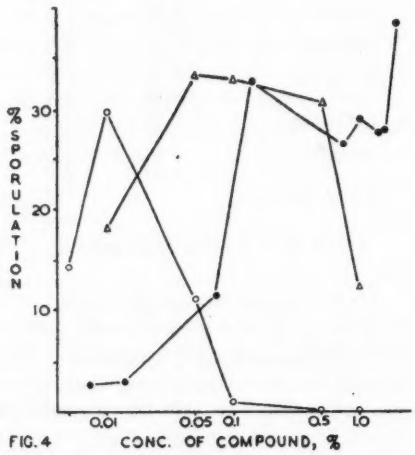

FIG. 4

FIG. 3. Yields of asci after seven to nine days on water agar slants containing three hexoses and two hexose phosphates. ○ = glucose; ● = glucose-1-phosphate; □ = fructose; ■ = fructose-1,6-diphosphate; △ = mannose. For comparison, yields were: 2.2% with water agar alone; 26.8% with 0.1% sodium acetate; 3.5% with 0.1% KH_2PO_4; 4.1% with 0.1% KH_2PO_4 + 0.1% glucose. FIG. 4. Yields of asci after seven to eight days on water agar slants containing three compounds. ○ = glucose; ● = glucose-1-phosphate; △ = sodium acetate. For comparison, yield with water agar alone was 5.5%.

Stimulation of Sporulation by Aspartate

It has recently been postulated (19) that acetate is involved in the biosynthesis of aspartic acid in yeast, which suggests that the latter compound might stimulate sporulation.

An experiment was done using as sporulation medium distilled water + five concentrations of aspartic acid, neutralized with sodium hydroxide. The results, shown in Table VIII, indicate that aspartate stimulates sporulation, especially at the more dilute concentrations. Of interest is the fact that most of the asci formed in aspartate contained only two spores.

TABLE VIII

SPORULATION OF STRAIN FJ IN SOLUTIONS OF SODIUM ASPARTATE

Concentration of aspartic acid previous to neutralization, %	Yield of asci, %	Yield of 3- and 4-spored asci, %
0.1	0 *	0 *
0.05	8.1	0.4
0.025	5.4	0
0.013	16.9	0.7
0.0063	21.0	2.0
0.0	3.8	0.1

** Each figure based on an examination of 1000 cells; 200 from each of five vials.*

Stimulation of sporulation in *S. cerevisiae* by aspartic acid was evidently first observed by Shannon (14), who obtained 60% asci in a lactose + salts solution containing 0.08% aspartic acid. Kleyn (12) added aspartic acid (0.15%) as nitrogen source to an agar medium on which a period of vegetative growth occurred prior to sporulation. Most of the asci that formed were one- or two-spored. However, since Kleyn's medium contained biotin, various salts, and two carbon sources (acetate and glucose) in low concentrations, it is difficult to decide from his data whether aspartic acid alone is stimulating and responsible for the low numbers of three- and four-spored asci.

Effect of Other Compounds on Sporulation

It has been found (16, 1) that salts of acetic acid are usually more stimulating to yeast sporulation than the acid itself, and this has been confirmed in the present study with other yeast strains (FJ, L, B). The writers have also observed that ammonium acetate in 1% or 0.1% concentration inhibits sporulation, although this has not been invariable. At a concentration of 0.01%, however, this compound is stimulating. Elder (9) found that as little as 0.08% ammonium nitrate prevented sporulation when included in acetate–glucose sporulation medium. In 1916 Saito (13) reported that ammonium salts had a depressing effect on the sporulation of cells of *Saccharomyces mandshuricus* spread on moistened gypsum blocks.

TABLE IX

COMPOUNDS THAT DID NOT STIMULATE SPORULATION

Compound	Concentrations employed, %	Presporulation medium	Yeast strains
Sodium formate	0.01, 0.05, 0.1, 0.5, 1.0, 2.0	5% Glucose nutrient agar	FJ, L, B
Sodium propionate	"	"	"
Propionic acid	"	"	"
Sodium oxalate	0.01, 0.1, 1.0	"	FJ
Oxalic acid	"	"	"
Sodium citrate	0.01, 0.1, 1.0	"	"
Citric acid	0.01, 0.1, 1.0	"	"
Aminoacetic acid	0.01, 0.05, 0.1, 0.5, 1.0, 2.0	"	FJ, L, B
Glutaric acid	0.01, 0.05	5% Glucose nutrient agar	FJ
		5% Glucose nutrient agar + 1% yeast extract	
Indole-3-acetic acid	0.000,001, 0.000,01, 0.0001, 0.001	5% Glucose nutrient agar	FJ, L, B
Acetaldehyde	0.01, 0.05, 0.1, 0.5, 1.0	5% Glucose nutrient agar	FJ
		5% Glucose nutrient agar + 1% yeast extract	
Ethyl alcohol	0.01, 0.05, 0.1, 0.5, 1.0	5% Glucose nutrient agar + 1% yeast extract	"
Histamine	0.01, 0.05	5% Glucose nutrient agar	"
		5% Glucose nutrient agar + 1% yeast extract	
Glucosamine	0.01, 0.05	5% Glucose nutrient agar	"
		5% Glucose nutrient agar + 1% yeast extract	

In Table IX are listed a number of compounds that have not proved stimulating to sporulation. On one occasion sodium propionate gave some evidence of stimulation, but attempts to confirm it were unsuccessful. Stantial (16) did not obtain sporulation with sodium propionate. She also tested oxalic acid, tartaric acid, sodium potassium tartrate, and sodium butyrate without observing stimulation. She found that in water containing 0.01% ethyl alcohol, 10% "bad ascs" developed, whereas no asci formed in 1% alcohol. The latter concentration inhibited sporulation when added to a sporulation medium containing 0.02% glucose and 0.2% acetate. Adams and Miller (3) reported that ethyl alcohol vapors inhibited sporulation on acetate–glucose agar. In the present study an experiment was done in which two yeast strains that produced fairly abundant asci on water agar were spread on slants of this medium containing three concentrations of ethyl alcohol. The results, shown in Table X, indicate that ethyl alcohol also inhibits sporulation in strains that can sporulate on water agar.

TABLE X

SPORULATION OF TWO YEAST STRAINS ON WATER AGAR SLANTS CONTAINING ETHYL ALCOHOL

Concentration of ethyl alcohol, %	Sporulation, %	
	Strain F54A	Strain F54B
1.0	0	2.0
0.1	1.6	11.2
0.01	23.4	29.7
0	22.0*	36.0*

** Each figure based on an examination of 600 cells; 200 from each of three tubes.*

Discussion

In studying the stimulation of fungus growth or sporulation by a substance it is of importance to relate the response to some influence on metabolism. The fact that, of all the compounds tested in this study, only assimilable sugars, glucose-1-phosphate, fructose-1,6-diphosphate, acetate, and aspartate stimulated sporulation may be taken to suggest that these compounds exert their effect on sporulation through their connection with the reactions involved in carbohydrate metabolism under aerobic conditions. This view finds support in the work of Adams and Miller (3) who observed that oxygen stimulated sporulation in an acetate–glucose medium. Further, Tremaine and Miller (17) were able to show that the stimulation of sporulation of well-nourished cells by acetate solution was increased by pantothenate. This B vitamin is known to form part of the Coenzyme A molecule which is directly involved in acetate metabolism. Citrate, however, did not affect sporulation but this may be the result of an inability to pass the osmotic barrier. Conway

and Downey (6) found that the inner surface of the outer metabolic region of the cell was impermeable to this compound, or very slowly permeable. The foregoing contributions indicate the advisability of testing more compounds known to be involved in the pathways of carbohydrate metabolism in yeast. This study is now in progress and will be reported on at a later time.

Some important differences are apparent between the chemical conditions that stimulate yeast sporulation and those that favor yeast growth. (*a*) Optimum sporulation occurs at a concentration of hexose (one part in 2000, or sometimes less) that is much lower than the optimum for growth. (*b*) Abundant sporulation also occurs in acetate concentrations of a similar order (e.g., one part in 1000). However, growth of the yeast studied was not clearly apparent on slants of Wickerham's (20) yeast nitrogen base + 2% agar with 1% acetate as the carbon source. Wang *et al.* (18) found that, although it was assimilated by bakers' yeast, acetate did not allow significant growth. (*c*) Growth is abundant in a chemically-defined medium containing 0.5% ammonium nitrate as nitrogen source. But, as pointed out above, 0.08% ammonium nitrate has been found to inhibit sporulation in an acetate–glucose medium (9), and sporulation was seldom observed by the writers in 0.1% ammonium acetate. (*d*) Sporulation is almost totally suppressed by ethyl alcohol in a concentration (1%) that does not inhibit growth.

The observation that low sporulation yields accompany low cell population densities in acetate solution is possibly due to a loss of coenzymes or other essential metabolites into the solution. When the cell population density is optimum, these metabolites may reach a sufficiently high concentration in the environment to allow active sporulation. On the other hand, the decline in yields at high cell population densities is perhaps because the volume is insufficient to dilute certain diffusible metabolites to a concentration low enough to permit sporulation. Compounds yielding ammonia, for example, may remain in high enough concentration to inhibit sporulation. If acetate supply were the limiting factor at high cell population densities, one would expect the optimum density in Fig. 1 to shift to the right at the higher concentrations of acetate.

S. cerevisiae provides a useful experimental subject for investigating the effect of nutritional factors on ascospore formation because no phase of multicellular growth need intervene between application of a stimulus and the appearance of spores. Products of growth metabolism therefore do not accumulate and alter the environment. This allows the effects of individual factors on ascospore formation to be more precisely studied than with most other Ascomycetes.

Acknowledgment

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References

1. ADAMS, A. M. A convenient method of obtaining ascospores from bakers' yeast. Can. J. Research, C, 27 : 179–189. 1949.
2. ADAMS, A. M. A comparative study of ascospore formation by 43 yeast cultures. Can. J. Research, F, 28 : 413–416. 1950.
3. ADAMS, A. M. and MILLER, J. J. Effect of gaseous environment and temperature on ascospore formation in *Saccharomyces cerevisiae* Hansen. Can. J. Botany, 32 : 320–334. 1954.
4. BARKER, B. T. A. On spore-formation among the Saccharomycetes. J. Inst. Brewing, 8 : 26–76. 1902.
5. BUSTON, H. W., JABBAR, A., and ETHERIDGE, D. E. The influence of hexose phosphates, calcium and jute extract on the formation of perithecia by *Chaetomium globosum*. J. Gen. Microbiol. 8 : 302–306. 1953.
6. CONWAY, E. J. and DOWNEY, M. An outer metabolic region of the yeast cell. Biochem. J. (London), 47 : 347–355. 1950.
7. EATON, N. R. and KLEIN, H. P. The oxidation of glucose and acetate by *Saccharomyces cerevisiae*. J. Bacteriol. 68 : 110–116. 1954.
8. ELDER, M. L. Heat treatment and the sporulation of yeast. M.A. Thesis, (unpublished). Department of Chemistry, University of Toronto, Toronto, Ont. 1933.
9. ELDER, M. L. The sporulation of bakers' yeast. Ph.D. Thesis, (unpublished portion). Department of Chemistry, University of Toronto. Toronto, Ont. 1937.
10. HAWKER, L. E. Stimulation of the formation of perithecia of *Melanospora destruens* Shear by small quantities of certain phosphoric esters of glucose and fructose. Ann. Botany, 12 : 77–79. 1948.
11. HENRY, B. S. The viability of yeast cultures preserved under mineral oil. J. Bacteriol. 54 : 264. 1947.
12. KLEYN, J. G. A study of some environmental factors controlling sporulation of yeast using a new sporulation medium. Wallerstein Labs. Communs. 17 : 91–102. 1954.
13. SAITO, K. Untersuchungen über die chemischen Bedingungen für die Entwicklung der Fortpflanzungsorgane bei einigen Hefen. J. Coll. Sci. Imp. Univ. Tokyo, 39 : 1–73. 1916.
14. SHANNON, B. M. Heat treatment and the sporulation of yeast. Chemical conditions which bring about the formation of giant cells in different yeasts. M.A. Thesis, (unpublished). Department of Chemistry, University of Toronto, Toronto, Ont. 1933.
15. STANTIAL, H. The sporulation of yeast. Trans. Roy. Soc. Can. III, 22 : 257–261. 1928.
16. STANTIAL, H. The sporulation of yeast. II. Trans. Roy. Soc. Can. III, 29 : 175–188. 1935.
17. TREMAINE, J. H. and MILLER, J. J. Effect of six vitamins on ascospore formation by an isolate of bakers' yeast. Botan. Gaz. 115 : 311–322. 1954.
18. WANG, C. H., LABBE, R. F., CHRISTENSEN, B. E., and CHELDELIN, V. H. Utilization of C^{14}-labelled pyruvate and acetate by yeast. J. Biol. Chem. 197 : 645–653. 1952.
19. WANG, C. H., THOMAS, R. C., CHELDELIN, V. H., and CHRISTENSEN, B. E. Conversion of acetate and pyruvate to aspartic acid in yeast. J. Biol. Chem. 197 : 663–667. 1952.
20. WICKERHAM, L. J. Taxonomy of yeasts. U. S. Dept. Agr. Tech. Bull. 1029. 1951.

PIGMENT PRODUCTION IN SKIM MILK BY ACTINOMYCETES IN RELATION TO POTATO SCAB[1]

By E. B. Vaisey[2], J. A. Carpenter[3], and R. G. Atkinson[4]

Abstract

This investigation confirms the reports of other workers that most actinomycetes isolated from potato scab lesions will form a dark brown ring in skim milk, and this characteristic may be positively correlated with the ability of these organisms to cause scab on potatoes. Of 39 "brown ring" isolates from potato scab lesions, 37 produced scab, whereas none of the 30 isolates that did not form a brown ring were pathogenic. This relationship did not exist, however, when studies were extended to include actinomycetes from the soil. Only six of the 43 soil isolates that produced a brown ring in skim milk were able to cause scab.

Introduction

Certain phases of research on potato scab control might be greatly accelerated if the pathogenic organisms, *Streptomyces scabies* (Thaxter) Waksman & Henrici, could be easily identified. To date the most promising method of distinguishing *S. scabies* from certain nonpathogenic actinomycetes is the skim milk test of Taylor and Decker (12). Their work was based mainly on isolates from potato scab lesions and showed that the production of a dark brown ring of surface growth in separated skim milk was correlated perfectly with the ability of actinomycetes to produce potato scab. However, as most of their cultures were isolated from scab lesions, it does not necessarily follow that the relationship would extend to the more varied actinomycete population of the soil. Accordingly, the data reported herein represent examinations of the pathogenicity of brown ring actinomycetes isolated both from potato scab lesions and from soil.

It is to be noted that throughout this communication, the designation *S. scabies* is used for those cultures which cause typical scab on the susceptible Katahdin variety. Otherwise the more general term "actinomycete" is used.

Materials and Methods

Actinomycetes from potato scab lesions were obtained from tubers harvested in 1949 from fields located in seven potato growing areas in Ontario. The method of isolation closely followed that of Taylor (11) with the exception that the organisms were plated out on soil extract agar instead of on Waksman's egg-albumen agar. In each series of isolations five to seven

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actinomycete colonies showing the greatest differences in macroscopic characteristics were transferred to soil extract agar slants. In this way 128 actinomycetes were obtained and their pigment production was tested in sterilized skim milk.

The actinomycetes isolated from soil were chosen by lot from a culture collection of some 350 isolates that produced a brown ring in skim milk. These actinomycetes had been isolated from the soil of three potato growing areas by nonselective methods described by Lochhead and Chase (6).

The inoculum of the actinomycetes selected for pathogenicity trials was obtained by flooding plates of glycerol–asparagine agar with spore suspensions in a 1 : 5000 solution of Tween-20*. After good growth had occurred, the agar from two of these plate cultures was cut into small squares and thoroughly mixed with sterilized soil from the upper portion of a 7- or 9-in. pot. The soil had been previously sterilized in the pots by steam under 15 lb. pressure for two hours.

In the pathogenicity trials with the isolates from scab lesions, scab-free whole potato tubers of the susceptible variety Katahdin were surface sterilized by immersion for half an hour in a 1 : 1000 mercuric chloride solution before being planted in the infested soil. The soil used was a light sandy loam from a field that consistently produced severely scabbed potatoes. These trials were conducted in four series, one begun in February, two in March, and one in May, 1950. Water was added daily to the pot saucer in order to keep the surface of the soil dry and to reduce the danger of air-borne contaminants becoming established.

In the pathogenicity trials of the actinomycetes from soil a similar procedure was employed with some modification. Since environmental factors such as the water holding capacity of the soil influence the initiation and severity of potato scab (2, 5), these trials were conducted with two different types of soil. One was the sandy loam previously mentioned and the other a 1 : 5 mixture of vermiculite and greenhouse potting soil. In this test the tubers were surface sterilized either by mercuric chloride or by a commercial formalin dip (three minutes in a 2% solution of commercial formalin at 50° C.). These trials were conducted from June to August of 1953. Water was added every other day to the pot saucers.

Results

About 54% of the 128 actinomycetes isolated from potato scab lesions produced a dark brown surface ring in tubes of sterilized skim milk. The next largest group, comprising about 26% of the isolates, failed to develop any pigment. The remaining 20% formed various pigments, mostly pink or salmon, and yellow.

All of the 70 actinomycetes that caused brown ring could be placed in two groups on the basis of their cultural characteristics on glycerol–asparagine

* *A wetting agent obtained from the Atlas Powder Company, Wilmington, Del.*

agar slants. Both groups produced abundant "Smoke Gray"* aerial mycelium. One group, however, produced colonies having a "Tilleul Buff" to "Avellaneous" undersurface but caused little or no discoloration of the agar. The second group developed a much more deeply pigmented undersurface ranging from "Tawny Olive" to "Natal Brown" and colored the agar "Cinnamon". This more or less homogenous collection of isolates that caused brown ring is in marked contrast to the heterogeneous assemblage of the group that did not cause brown ring. None of the latter group could be placed in either of the two cultural groups of "brown ring" actinomycetes. This evidence strongly suggests that the "brown ring" isolates from the scab lesions represented one species, namely *S. scabies*. On the other hand, the isolates that did not cause brown ring represent various species and probably different genera.

In the pathogenicity trials (Table I) 39 of the 70 "brown ring" cultures isolated from scab lesions were tested. Thirty-seven caused scab, while two isolates proved to be nonpathogenic. No scab, or only a trace, appeared on tubers in the control pots, except for tubers in the trials initiated in May. Tubers in both control pots in this series showed 2% scab but this amount was much less than that produced by the pathogenic isolates tested in this series.

TABLE I

PATHOGENICITY TRIALS OF "BROWN RING" AND "NON-BROWN RING" ACTINOMYCETES, ISOLATED FROM POTATO SCAB LESIONS AND FROM THE SOIL

	Actinomycetes from potato scab lesions		Actinomycetes from the soil
	"Brown ring" cultures	"Non-brown ring" cultures	"Brown ring" cultures
Number of cultures tested for pathogenicity	39	30	43
Number of cultures causing potato scab	37	0	6

In contrast to the pathogenicity of the brown ring isolates, 29 of the 30 "non-brown-ring" actinomycetes from isolated scab lesions caused either no scab or only a trace similar to that occurring in some of the control pots. An average of 19% scab occurred in the two pots inoculated with one of the isolates that did not cause brown ring. In a second test, however, failure of this isolate to produce scab suggests that contamination was probably responsible for the first result.

Of the actinomycetes isolated from soil, only six of the 43 "brown ring" isolates tested proved capable of producing a significant amount of scab on the highly susceptible Katahdin tubers. No scab or only a trace occurred

* *Ridgway, R. Color standards and color nomenclature. Washington, D.C.*

in some of the control pots. The scabbed area produced by the six pathogenic actinomycetes was, in general, similar both in the vermiculite potting soil mixture, and in the sandy loam soil.

Discussion

The results reported here show that the brown ring test of Taylor and Decker indicates pathogenicity only when applied to actinomycetes isolated from potato scab lesions. It failed to separate pathogenic from nonpathogenic cultures when these were isolated from the soil. The fact that all actinomycetes producing a brown ring on skim milk also produced brown pigments on a medium containing tyrosine and asparagine as the nitrogen source suggests that these are similar in origin; that is they are melano-pigments resulting from the metabolism of tyrosine, or protein fragments containing tyrosil groups. Evidence for the oxidation of tyrosil groups by tyrosinase is given by Sizer (9). If this is the case then the failure of the "brown ring" test could have been anticipated for there are numerous references to soil actinomycetes, some nonpathogenic, which are able to produce brown pigments on media containing protein, tyrosine, or both substances (1, 3, 4, 10, 14). Unfortunately since there are no specific tests for melanin, it is impossible to prove directly that these pigments are of similiar origin to that produced in skim milk.

In spite of the fact that not all "brown ring" actinomycetes are pathogens, other workers have reported that all pathogenic cultures which they have investigated produce brown pigment, when cultured in skim milk (12), on media containing protein (7), and on media containing tyrosine (1). This cannot be entirely reliable because Millard and Burr (8) have described actinomycete species which cause potato scab but did not produce brown pigments. Furthermore preliminary work on mutant strains of *S. scabies* suggests that brown ring formation on skim milk is not associated with the ability to produce scab on potatoes (13).

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References

1. AFANASIEVE, M. M. Comparative physiology of actinomycetes in relation to potato scab. Univ. Nebr. Agr. Exptl. Sta. Research Bull. 92. 1937.
2. DIPPENAAR, B. J. Environment and seed treatment in relation to common potato scab. (Abstract) Phytopathology, 23 : 9. 1933.
3. DRECHSLER, C. Morphology of genus Actinomyces. Botan. Gaz. 67 : 147–164. 1919.
4. HOOKER, W. J. Parasitic action of *Streptomyces scabies* on roots of seedlings. Phytopathology, 39 : 442–462. 1949.

5. JAQUES, R. P. The effect of carbamates on the soil microflora with reference to Actinomycetes and possible application in control of potato scab. M.S.A. Thesis, School of Graduate Studies, The University of Toronto, Toronto, Ont. May, 1954.
6. LOCHHEAD, A. G. and CHASE, F. E. Qualitative studies of soil microorganisms. V. Nutritional requirements of the predominant bacterial flora. Soil Sci. 55 : 185-195. 1943.
7. LUTMAN, B. F. and CUNNINGHAM, G. C. Potato scab. Vt. Agr. Exptl. Sta. Bull. 184. 1914.
8. MILLARD, W. A. and BURR, S. A study of twenty-four strains of actinomyces and their relation to types of common scab of potato. Ann. Appl. Biol. 13 : 59–64. 1926.
9. SIZER, I. W. The action of tyrosinase on certain proteins and products of their autolysis. J. Biol. Chem. 169 : 303–311. 1947.
10. SKINNER, C. E. The "tyrosinase reaction" of the actinomycetes. J. Bacteriol. 35 : 415–424. 1938.
11. TAYLOR, C. F. A method for the isolation of actinomycetes from scab lesions on potato tubers and beet roots. Phytopathology, 26 : 387–388. 1936.
12. TAYLOR, C. F. and DECKER, P. A correlation between pathogenicity and cultural characteristics in the genus actinomycetes. Phytopathology, 37 : 49–58. 1947.
13. VAISEY, E. B. An investigation of actinomycetes isolated from the soil and their ability to cause potato scab. M.S.A. Thesis, School of Graduate Studies, The University of Toronto, Toronto, Ont. November, 1953.
14. WAKSMAN, S. A. Cultural studies of species of actinomycetes. Soil Sci. 8 : 71–215. 1919.

THE BACTERIAL DISSIMILATION OF NAPHTHALENE[1]

By J. F. Murphy[2] and R. W. Stone

Abstract

Data are presented indicating that the major pathway for the oxidation of naphthalene by a strain of *Pseudomonas* occurs via salicylic acid, which is further oxidized through catechol to β-ketoadipic acid. On the basis of growth, simultaneous adaptation, and cell-free extract experiments, the following compounds are regarded as unlikely intermediates in naphthalene dissimilation: 1,4-naphthoquinone, α-naphthol, β-naphthol, 1,3-dihydroxynaphthalene, 2,3-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, phenol, *trans-o*-hydroxycinnamic acid, and phthalic acid. Evidence was found that a second pathway of naphthalene oxidation produces 1,2-naphthoquinone. 1,2-Dihydroxynaphthalene was found to be non-enzymatically converted to 1,2-naphthoquinone. The 1,2-naphthoquinone was not further metabolized and was found to be responsible for the characteristic brown to reddish orange color of the culture medium. Omission of $FeCl_2$ and $MgSO_4$ from the basal medium prevented the formation of salicylic acid but did not interfere with the production of 1,2-naphthoquinone.

Introduction

In recent years there has been a widespread interest in the microbiological dissimilation of aromatic compounds. New chemical and microbiological techniques have made it possible to establish the mechanism of degradation for a number of aromatic compounds (10). For the most part, these metabolic pathways have been determined for benzene derivatives only, but studies on amino acids such as tryptophane have shown that these pathways may be common for a variety of aromatic compounds.

It was known for many years that naphthalene was dissimilated by microorganisms (6, 14, 15), but until recently nothing had been known about the metabolic pattern of this breakdown. In 1943 Strawinski and Stone (12, 13) studied the optimum conditions for the dissimilation of naphthalene by a strain of *Pseudomonas* and found that salicylic acid could be isolated from the culture medium. More recently Walker and Wiltshire (19) isolated a naphthalene diol identified as D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from cultures of an unidentified bacterium growing on a naphthalene substrate. The investigators also isolated salicylic acid from growing cultures and found that resting cells showed an immediate oxygen uptake with the D-"trans"-diol, salicylic acid, and catechol. They also obtained evidence of a small amount of oxidation with coumarin, 1,2-naphthoquinone, and 1,2-dihydroxynaphthalene. In a later paper Treccani, Walker, and Wiltshire (16) have extended their studies to include three additional *Pseudomonas* strains and a strain of *Nocardia*. In all of these cultures D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene, salicylic acid, and catechol appeared to be intermediate oxidation products. Washed cells of these organisms also oxidized α- and β-naphthol.

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In this study we have further investigated the bacterial dissimilation of naphthalene and compared the degradation of this compound with the metabolic pathways believed to be involved during the breakdown of the benzene derivatives.

Experimental Methods

Using a soil enrichment technique, several strains of bacteria capable of growing on naphthalene were isolated. These bacteria were all found to be members of the genus *Pseudomonas* and resembled the hydrocarbon-utilizing members of this genus, *Pseudomonas arvilla* and *Pseudomonas rathonis*, described in Bergey's Manual of Determinative Bacteriology (3). One strain, designated N_1, was selected for use throughout the course of this study.

The studies were carried out in both 2-liter air-stirred fermenters and 5-gal. carboys fitted with a mechanical stirring device and aluminum aeration tubes. For some experiments 500 ml. Erlenmeyer flasks containing 100-125 ml. of medium were incubated on a reciprocating shaker. The circulating fermenters and shaken flasks were held at room temperature (from 23-25° C.) whereas the experiments using the 5-gal. carboys were held at 30° C.

The basal mineral salts medium had the following composition: 4 gm. NH_4NO_3, 1 gm. K_2HPO_4, 0.5 gm. KH_2PO_4, 0.5 gm. $MgSO_4 \cdot 7H_2O$, 0.2 gm. $MnCl_2 \cdot 4H_2O$, 0.05 gm. $CaCl_2 \cdot 2H_2O$, 0.05 gm. $FeCl_2 \cdot 4H_2O$, 2 gm. $CaCO_3$, and 1 liter tap water. Since preliminary experiments showed that a small amount of yeast extract aided in the biological attack of naphthalene, usually 0.1% dehydrated yeast extract was added to the basal medium. The concentration of naphthalene routinely used was 1.0%.

Ultraviolet spectrophotometric analyses were run on a Model DU Beckman Spectrophotometer, with 0.2 mm. slit width, cell depth of 0.96 cm., and either 95% ethyl alcohol or distilled water as solvents. A quantitative colorimetric test, based on the reaction between $FeCl_3$ and salicylic acid, was used to determine the salicylic acid content of the fermentation medium during naphthalene dissimilation.

Manometric experiments were carried out at 30° C. using both whole cells and cell-free extracts. The common Warburg "Direct Method" outlined by Umbreit, Burris, and Stauffer (17) was used to determine the oxygen uptake on naphthalene and other aromatic hydrocarbons. Cell-free extracts were prepared by disintegration at 9 kc./sec. in a Raytheon Sonic Oscillator for 60 min. The oscillator was operated with the plate voltage of 135 volts, and ice water was circulated through the outer chamber of the container to maintain a temperature of 10° C. or lower in the cell suspension during treatment.

Results

Growth on Naphthalene

The oxidation of naphthalene, whether carried out in air-stirred fermenters, 12-liter fermenters, or shaken flasks caused the colorless medium to darken as the fermentation proceeded. After 24 hr. the medium was light brown, and from 48 to 72 hr. the pigmentation varied from an orange brown to a

brownish black. With 1.0% initial concentration of naphthalene, particles were still visible in the medium after 72 hr. incubation. During the first 12 hr. the pH dropped sharply from 7.0 to between 5.5 and 5.0. This pH level was maintained until toward the end of the incubation period when it had a tendency to rise. Under normal conditions, small aliquots of the fermentation liquor gave a purple color with the $FeCl_3$ reagent after 12 to 20 hr. incubation. After 30 hr., or sooner depending upon the time the positive test was first obtained, the reaction was negative. However, a negative test did not necessarily indicate the end of bacterial growth. Fig. 1 demonstrates that cells grown on naphthalene may produce a "diauxie" type growth curve similar to that described by Monod (9). It is interesting to note that the decrease in cell count occurred during the period when the positive $FeCl_3$ test was obtained, thus corresponding to the period when excess salicylic acid was being formed.

Isolation and Identification of Salicylic Acid

Using the method of Strawinski and Stone (13), a white crystalline solid was isolated from the fermentation liquor at the time that the $FeCl_3$ reaction was most intense. The properties of the isolated compound were identical with those of salicylic acid given by Huntress and Mulliken (5). Fig. 2 shows the ultraviolet absorption curves of the isolated material and a known sample of salicylic acid, and leaves little doubt that the isolated compound was definitely salicylic acid.

Since the intensity of the purple color with $FeCl_3$ is proportional to the concentration of the salicylic acid, a colorimetric semiquantitative test was devised to measure the amount of salicylic acid that was produced during naphthalene dissimilation. The amount of salicylic acid which was produced during a typical fermentation is shown in Fig. 3. It was also apparent that salicylic acid comprised a major portion of the ether-extractable solids formed from naphthalene as found previously by Strawinski and Stone.

Growth experiments using salicylic acid as a substrate, in concentrations ranging from 0.005 to 0.25%, showed that the *Pseudomonas* strain N_1 was able to utilize salicylic acid at all concentrations tried.

Effect of Elimination of Ferrous and Magnesium Salts from the Basal Medium

When the ferrous and magnesium salts were eliminated from the basal medium prepared with distilled water, a marked change occurred in the pattern of naphthalene dissimilation previously described. Fig. 4 shows the results obtained in this medium. Omission of $MgSO_4$ retarded the lowering of pH, and the omission of both salts resulted in the pH remaining near neutral. Omission of either or both salts also resulted in a negative $FeCl_3$ test, indicating that no salicylic acid was formed. In contrast to the color change occurring during oxidation with the normal basal medium, the following color change occurred when a deficient medium was used: after 9 to 12 hr. of incubation the medium became light yellow, darkening as the fermentation progressed, changing to orange-red in 24 to 26 hr.

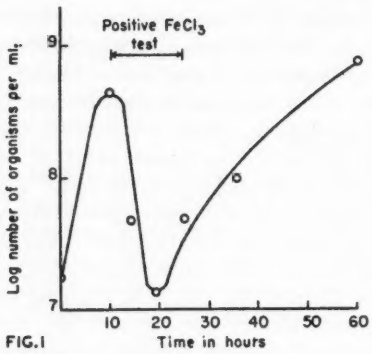

FIG. 1

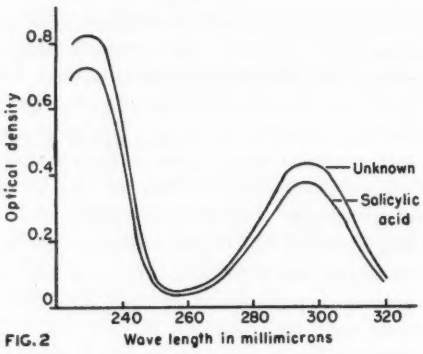

FIG. 2

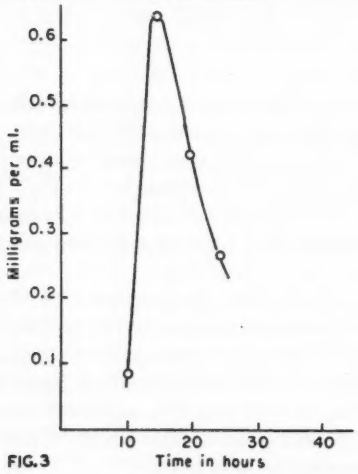

FIG. 3

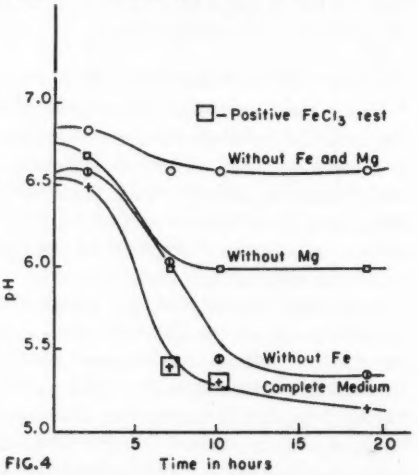

FIG. 4

FIG. 1. Bacterial growth on naphthalene. FIG. 2. Absorption spectra of salicylic acid and unknown acid isolated from growth medium. FIG. 3. Salicylic acid produced during naphthalene oxidation. FIG. 4. Effect of eliminating Fe^{++} and Mg^{++} from the medium.

Isolation and Identification of 1,2-Naphthoquinone

The material responsible for the orange-red pigmentation in the ferrous- and magnesium-deficient medium was isolated by extraction of the medium with ethyl ether at pH 2.0. The material was recrystallized from an ethyl alcohol – water mixture and found to be a reddish-brown solid. The isolated material upon ignition burned with a yellow smoky flame. At 120° C. the material blackened and decomposed. The resultant tarry mass melted at 138-140° C. An elemental analysis showed nitrogen, sulphur, and the halogens to be absent. The compound was insoluble in water, soluble in 5% NaOH, 5% $NaHCO_3$, and concentrated H_2SO_4. It produced a green coloration when dissolved in concentrated H_2SO_4. The compound gave a positive Edlefsen reaction and a solid derivative with phenylhydrazine. These

characteristics of the unknown compound indicated that it was 1,2-naphthoquinone. Ultraviolet absorption curves of the phenylhydrazine derivative obtained from a known sample of 1,2-naphthoquinone and the phenylhydrazine derivative of the isolated compound were identical as shown in Fig. 5. The melting point of the known phenylhydrazine derivative was 129° C. and that of the phenylhydrazine derivative obtained from the isolated material was 130° C. The mixed melting point obtained from both derivatives was 130° C.

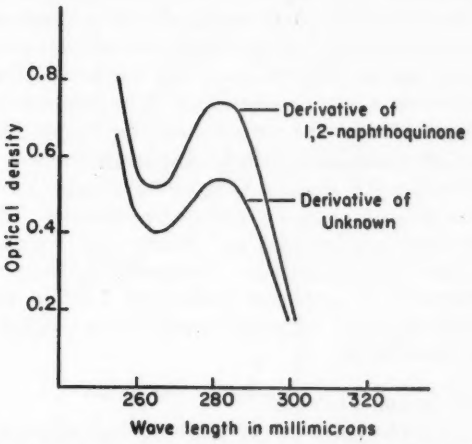

FIG. 5. Absorption spectra of phenylhydrazine derivatives of 1,2-naphthoquinone and unknown from the medium.

Growth Experiments on 1,2-Naphthoquinone and Other Aromatic Compounds

Experiments with shaken flasks and the aerated circulating fermenters were run with 1,2-naphthoquinone as a substrate. The results indicated that 1,2-naphthoquinone was not utilized by the culture that produced it. Bacterial counts showed no evidence of bacterial growth and no significant pH changes occurred during the progress of the experiment. A negative $FeCl_3$ test was obtained on all samples tested, and spectrophotometric analyses of the medium as the experiment progressed showed no significant change in the initial absorption curve given by the compound. Experiments using a mixed substrate of 0.1% 1,2-naphthoquinone and 0.9% naphthalene indicated that the quinone at this concentration inhibited the breakdown of naphthalene. Shaken flask experiments using a mixed substrate of 0.25% naphthalene and 0.03% or less of the quinone did not show any inhibition of the ability of the organism to form salicylic acid.

Shaken flask experiments were run using as substrates for growth various aromatic compounds that might be possible intermediates in the breakdown of naphthalene to salicylic acid. A concentration of 0.2% was used for all compounds studied except 1,2-dihydroxynaphthalene which was used at a concentration of 0.08%. The following compounds were tested: α-naphthol,

β-naphthol, 1,4-naphthoquinone, naphtholresorcinol, 2,3-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, and *trans-o*-hydroxycinnamic acid. There was no evidence for the formation of salicylic acid from the action of the *Pseudomonas* culture on any of the compounds tested other than naphthalene.

Chemical Detection of Other Intermediates

Jacobs (6) studied the dissimilation of naphthalene by pure cultures of soil bacteria and found that the culture medium gave a positive test for phthalic acid. The colorimetric test used by Jacobs was run on the fermentation liquor at various time intervals and a positive test was obtained; however, phthalic acid could not be isolated from the medium. The test was also found to be positive on a known solution of 1,2-naphthoquinone; therefore, the compound responsible for the positive reaction reported by Jacobs in the fermentation medium could have been 1,2-naphthoquinone.

Walker and Wiltshire (19) isolated D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from growing cultures on a naphthalene substrate. Another group of investigators, Boyland and Levi (1) and Young (20), found this compound in the urine of rats fed naphthalene. Using the colorimetric technique of Boyland and Wiltshire (2) repeated tests were run on the fermentation medium at periodic intervals. A positive test for this diol was not obtained on any of the samples tested.

Manometric Studies (with Whole Cells)

The oxidation of naphthalene in the Warburg apparatus was studied using 32, 16, 8, 4, and 2 μM. of naphthalene as a substrate. After 110 min. the flask contents were removed and $FeCl_3$, Rothera, and naphthalene diol tests were run on the contents. As shown in Fig. 6, the cells oxidized naphthalene at all levels of concentration used. The flasks containing 16 and 32 μM. of naphthalene gave positive Rothera and $FeCl_3$ tests. All flasks gave a negative test for the presence of D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene. Similar experiments were run using catechol, salicylic acid, and 1,2-naphthoquinone as substrates. The results presented in Fig. 7 show that cells grown on naphthalene were able to oxidize catechol and salicylic acid but not 1,2-naphthoquinone at the concentration used.

Manometric Studies with Cell-free Extracts

Preliminary experiments demonstrated that cell-free extracts could be prepared, by sonic disintegration of naphthalene-grown cells, which would oxidize both naphthalene and salicylic acid. Since cells grown on naphthalene showed rapid oxygen uptakes on salicylic acid and catechol, experiments were run using concentrations of 4 μM. per 3.5 ml. volume. As shown in Fig. 8, the extract oxidized both salicylic acid and catechol. At the end of the reaction time, the flask contents gave a positive Rothera test indicating that β-ketoadipic acid was formed.

The ability of the extract to oxidize aromatic compounds that might be possible intermediates in the oxidation of naphthalene was tested using 4 μM.

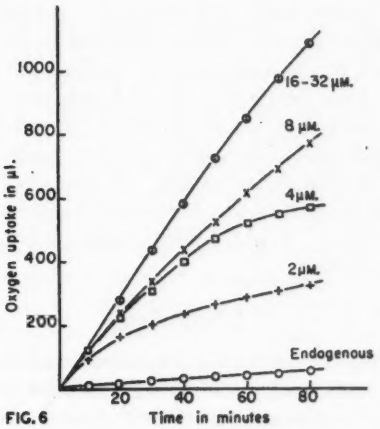

FIG. 6

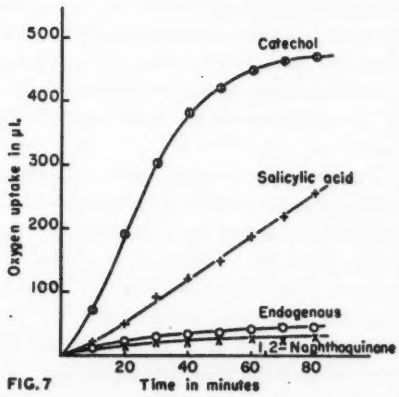

FIG. 7

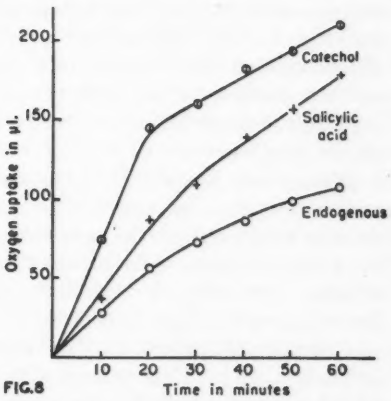

FIG. 8

FIG. 6. Oxidation of naphthalene by cell suspensions. FIG. 7. Oxidation of aromatic compounds by cells grown on naphthalene. Substrate conc. = 4 μM. FIG. 8. Oxidation of salicylic acid and catechol by a cell-free extract of cells grown in naphthalene. Substrate conc. = 4 μM.

per flask of the following substrates: α-naphthol, β-naphthol, naphthoresorcinol, 2,3-dihydroxynaphthalene, 1,5-dihydroxynaphthalene, 1,4-naphthoquinone, *trans-o*-hydroxycinnamic acid, phthalic acid, and phenol. None of these compounds showed any significant oxygen uptake over and above the endogenous respiration. Although the intact cells gave no evidence of attacking 1,2-naphthoquinone, an experiment was run with the cell-free extract to check the possibility that lack of permeability might explain the inability of the naphthalene-grown cells to attack 1,2-naphthoquinone. The quinone was used in concentrations varying from 0.2 to 8μM. per 3.5 ml. No demonstrable oxygen consumption by the extract was obtained with any of the concentrations of 1,2-naphthoquinone used.

Numerous experiments using 1,2-dihydroxynaphthalene as a substrate demonstrated that some oxygen uptake occurred in the presence or absence of the cell-free extract, but in either case the medium at the end of the reaction gave a negative Rothera and $FeCl_3$ test. Ether extraction of the reaction mixture removed a black tarry material which reacted with phenylhydrazine to give a solid derivative. This derivative was found to be identical with that obtained from 1,2-naphthoquinone. It appears likely that in neutral or slightly alkaline solutions 1,2-dihydroxynaphthalene was non-enzymatically oxidized to the quinone. The 1,2-dihydroxynaphthalene was found to be more stable in acid solution.

Discussion

In concurrence with the findings of Strawinski and Stone (13) and Walker and co-workers (16, 19), salicylic acid was isolated from the fermentation medium during the dissimilation of naphthalene. Studies with cell-free extracts demonstrated that cells grown on naphthalene possessed the necessary enzymes for the oxidation of salicylic acid. These experiments, plus the demonstration that salicylic acid was readily attacked by the *Pseudomonas* culture, provide further evidence that salicylic acid is an intermediate in a pathway of naphthalene oxidation that is common among members of the genus *Pseudomonas*.

Walker and Evans (18), using a vibrio-type organism, found that salicylic acid was oxidized via catechol and *cis–cis*–muconic acid to β-ketoadipic acid. It was found during the course of the present study that extracts of the naphthalene-grown cells oxidized salicylic acid to a compound giving an intense violet color with the Rothera reagent which has been used by various investigators (4, 7, 11) to determine the presence of β-ketoadipic acid. Thus, one compound formed by the oxidation of salicylic acid was probably β-ketoadipic acid. Experiments with cell-free extracts also demonstrated that catechol was oxidized to β-ketoadipic acid, judging from the Rothera test. Although catechol was not isolated from the fermentation medium, the presence of an active catechol-oxidizing enzyme system in cells grown on naphthalene indicates that naphthalene dissimilation may occur via catechol. On the contrary, experiments using phenol as a growth substrate gave no catechol-activity indicating that phenol was probably not involved in the breakdown. A positive Rothera test was also obtained during the course of manometric experiments with intact cells and relatively high concentrations of naphthalene. From the preceding evidence and from the results of other investigators (19) it is concluded that naphthalene dissimilation occurs through salicylic acid, catechol, *cis–cis*-muconic acid to β-ketoadipic acid. As pointed out by Kilby (8), β-ketoadipic acid breaks down to succinic acid which may be further oxidized via the terminal respiratory cycles to carbon dioxide.

During the bacterial dissimilation of naphthalene the medium gradually became colored and after 48 to 72 hr. showed a brown to reddish-orange

pigmentation. Spectrophotometric studies on the medium and isolation of a phenylhydrazine derivative of the compound responsible for the color showed it to be 1,2-naphthoquinone. Subsequent experiments using 1,2-naphthoquinone as a substrate demonstrated that this compound was not attacked by the *Pseudomonas* culture. Further experimentation demonstrated that a 0.1% concentration of β-naphthoquinone completely inhibited naphthalene dissimilation and that the presence of 4 μM. of the quinone was sufficient to show a marked effect on endogenous respiration. The toxic effect of β-naphthoquinone was also noted by Treccani, Walker, and Wiltshire (16). These investigators found that the quinone in 1 μM. amounts inhibited both the oxidation of α-naphthol and the endogenous respiration of naphthalene-grown cells. However, we found that during the course of shaken-flask experiments, using a mixed substrate of 0.25% naphthalene and 0.03% or less of β-naphthoquinone, there was no inhibition of naphthalene dissimilation. Thus it appears that although β-naphthoquinone is a toxic material, its presence in small amounts during the dissimilation of naphthalene can be tolerated until its gradual accumulation becomes completely inhibitory. When this quinone was first isolated, it was supposed that it would readily be broken down by the organism. Stanier (11) proposed that this type compound was the intermediate that occurred just prior to the splitting of the ring. In his work on the dissimilation of benzene derivatives, Stanier observed that rupture of the ring is always preceded by the introduction of two adjacent hydroxyl groups. The next step suggested was an enol–keto rearrangement with oxidative rupture of the ring between adjacent carbonyl groups. If aromatic ring splitting does occur in this manner, one would expect 1,2-naphthoquinone to be readily dissimilated. However, repeated experiments showed that this was not the case with our culture. The 1,2-naphthoquinone was found to be an apparent end product and not an intermediate in the oxidation of naphthalene.

It appears that the bacterial dissimilation of naphthalene may occur via at least two pathways, one going through salicylic acid to carbon dioxide, and the other to 1,2-naphthoquinone which builds up in the medium as a highly colored and somewhat inhibitory product. Evidence of two pathways was obtained when $FeCl_2$ and $MgSO_4$ were omitted from the basal medium. Omission of these salts inhibited the formation of salicylic acid but did not affect the production of 1,2-naphthoquinone.

Growth experiments were run using α- and β-naphthol, several dihydroxy naphthalenes, 1,4-naphthoquinone, and *trans-o*-hydroxycinnamic acid to determine whether any of these compounds would produce salicylic acid from naphthalene. Simultaneous adaptation and cell-free extract studies were also run using naphthalene derivatives as substrates to determine whether cells grown on naphthalene possessed the enzymes necessary to oxidize these compounds. On the basis of this work, none of the compounds, except 1,2-dihydroxynaphthalene, gave any evidence of being available as a substrate for the enzymes of the *Pseudomonas* cultures.

It was found that 1,2-dihydroxynaphthalene, under neutral to alkaline conditions, was autocatalytically oxidized to 1,2-naphthoquinone, regardless of the presence of cells or cell extracts.

Walker and Wiltshire (19) isolated D-*trans*-1,2-dihydro-1,2-dihydroxynaphthalene from a culture growing on naphthalene and demonstrated by adaptation studies that this "diol" was a possible intermediate in the breakdown on naphthalene. During the course of our investigation no evidence of the presence of this compound was obtained upon analysis of the culture medium at various time intervals. Since Walker and Wiltshire did not employ forced aeration and their organisms likely differed from the *Pseudomonas* used in our investigation, it is possible that under our conditions the "diol" was utilized as rapidly as it was formed and consequently was not detected in the medium.

References

1. BOYLAND, E. and LEVI, A. A. Metabolism of polycyclic compounds. I. Production of dihydrodihydroxyanthracene from anthracene. Biochem. J. 29 : 2679–2683. 1935.
2. BOYLAND, E. and WILTSHIRE, G. H. Metabolism of naphthalene by liver slices. Biochem. J. 53 : 424–426. 1953.
3. BREED, R. S., MURRAY, E. G. D., and HITCHENS, A. P. Bergey's manual of determinative bacteriology. 6th ed. The Williams & Wilkins Company. Baltimore, Maryland. 1948.
4. EVANS, W. C. Oxidation of phenol and benzoic acid by some soil bacteria. Biochem. J. 41 : 373–382. 1947.
5. HUNTRESS, H. A. and MULLIKEN, S. P. Identification of pure organic compounds. John Wiley & Sons, Inc. New York, New York. 1949.
6. JACOBS, S. E. The influence of antiseptics on the bacterial and protozoan population of greenhouse soils. Part I. Naphthalene. Ann. Appl. Biol. 18 : 98–135. 1931.
7. KILBY, B. A. The bacterial oxidation of phenol to beta-ketoadipic acid. Biochem. J. 43 : V. 1948.
8. KILBY, B. A. The formation of beta-ketoadipic acid by bacterial fission of aromatic rings. Biochem. J. 49 : 671–674. 1951.
9. MONOD, J. Recherches sur la croissance des cultures bactériennes. Hermann et Cie, Paris. 1942.
10. STANIER, R. Y. Problems of bacterial oxidative metabolism. Bacteriol. Revs. 14 : 179–192. 1950.
11. STANIER, R. Y. The bacterial oxidation of aromatic compounds. IV. Studies on the mechanism of enzymatic degradation of protocatechuic acid. J. Bacteriol. 59 : 527–532. 1950.
12. STRAWINSKI, R. J. and STONE, R. W. Conditions governing the oxidation of naphthalene and the chemical analysis of its products. J. Bacteriol. 45 : 16. 1943.
13. STRAWINSKI, R. J. and STONE, R. W. Biological oxidation of naphthalene. Can. J. Microbiol. 1 : 206–210. 1954.
14. TATTERSFIELD, F. The decomposition of naphthalene in the soil and the effect upon its insecticidal action. Ann. Appl. Biol. 15 : 57–80. 1928.
15. TAUSSON, V. O. Naphthalene as a carbon source for bacteria. Chem. Abstr. 23 : 408. 1928.
16. TRECCANI, V., WALKER, N., and WILTSHIRE, G. H. The metabolism of naphthalene by soil bacteria. J. Gen. Microbiol. 11 : 341–348. 1954.
17. UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. Manometric techniques and tissue metabolism. Burgess Publishing Company, Minneapolis, Minn. 1949.
18. WALKER, N. and Evans, W. C. Pathways in the metabolism of the monohydroxybenzoic acids by soil bacteria. Biochem. J. 52 : xxiii. 1952.
19. WALKER, N. and WILTSHIRE, C. H. The breakdown of naphthalene by a soil bacterium. J. Gen. Microbiol. 8 : 273–276. 1953.
20. YOUNG, L. The metabolic conversion of naphthalene to 1,2-dihydronaphthalene-1,2-diol. Biochem. J. 41 : 417–422. 1947.

INHIBITION OF THE XYLOSE ISOMERASE OF *PSEUDOMONAS HYDROPHILA* BY SULPHYDRYL REAGENTS AND THE EFFECT OF GLUTATHIONE[1]

By R. M. Hochster

Abstract

Xylose isomerase of *Pseudomonas hydrophila* has been found to be strongly inhibited by substances known to combine with or oxidize sulphydryl groups of proteins. Considerable variation exists in the effectiveness of these inhibitors. Complete enzyme inhibition was observed with 2×10^{-4} *M* *p*-chloromercuribenzoate and with *p*-quinone, with 3.3×10^{-2} *M* N-ethylmaleimide and with 0.3 *M* iodoacetate. Glutathione, added after the enzyme and the inhibitors had been in solution, reversed only the inhibition caused by *p*-chloromercuribenzoate. Xylose isomerase, therefore, depends for its activity upon the integrity of sulphydryl groups.

Introduction

Xylose isomerase, the specific enzyme which catalyzes the interconversion of D-xylose and of D-xylulose, has been described previously for *Pseudomonas hydrophila* (7, 8), *Lactobacillus pentosus* (14), and most recently for *Pasteurella pestis* (18).

In the early work (8), no significant inhibitory effects on the *P. hydrophila* enzyme were observed with a variety of potential inhibitors including iodoacetate and *p*-chloromercuribenzoate at final concentrations of 10^{-3} *M*. For these studies, the enzyme was obtained as an acetone–dried powder from cell-free, sonic extracts prepared with added glutathione.

In later work, however, cell-free extracts containing xylose isomerase were obtained by grinding the cells with alumina (12) without the addition of glutathione. In these preparations, it was found that the isomerase was, indeed, inhibited by sulphydryl reagents. The apparent absence of inhibition in early observations with sonic preparations was found to be due to their high content of protein which protected the enzyme against the potential inhibitors used.

Results of the re-investigation of isomerase inhibition with much lower protein concentrations are reported in this paper.

Materials and Methods

Enzyme Preparation

Sonic extracts of *P. hydrophila* cells were prepared as described previously (8). One important difference, however, was that the cells were suspended in 0.002 *M* bicarbonate solution (approximately pH 7.6) to which no glutathione was added. Acetone powders were prepared from the supernatant solution and used as described (8). The powder preparations were assayed for protein ($N \times 6.25$) by the Kjeldahl method of McKenzie and Wallace

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Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3674.
Note: The results of this investigation were presented at the meeting of the Royal Society of Canada, June 6-8, 1955 held at Toronto, Canada.

(13). Values ranging from 6.7–8.0 mgm. per 10 mgm. powder were obtained for different preparations. No correction for non-protein nitrogen was made, since the values obtained were very small and quite constant.

Chemical Substances

The compounds used and their suppliers were as follows: D-xylose (Pfanstiehl Chemical Co.), iodoacetic acid (Anachemia Ltd.), N-ethylmaleimide and *p*-chloromercuribenzoic acid (Bios Laboratories, Inc.), *p*-quinone (Eastman Kodak) freshly sublimed before use, monosodium glutathione (Schwarz Laboratories Inc.), and crystalline bovine plasma albumin (Armour and Co.).

Analytical Method

D-Xylulose was estimated by the colorimetric method of Dische and Borenfreund (4) using a Farrand interference filter (538 mμ) in a Lumetron colorimeter. Glutathione and the inhibitors did not interfere with color development at the concentrations used in these experiments.

Experimental Technique and Sampling

Incubations were carried out in conventional Warburg vessels at 27° C. for a period of two hours in air in a total volume of 3 ml. The assay system was composed of 0.013 *M* phosphate buffer (pH 7.5), acetone powder equivalent to 10 mgm. protein, inhibitors, and reduced glutathione at the concentrations indicated in the text, and 0.18 *M* D-xylose tipped into the side arm at zero time. Glutathione (when used) was added only after the enzyme and the inhibitor had been added to the vessels. A 30 min. preincubation of enzyme and inhibitor was always allowed before addition of the substrate.

At the end of the incubation, 1.5 ml. reaction mixture was removed from each vessel and added to a mixture of 1.0 ml. 20% trichloroacetic acid and 1.5 ml. water. The precipitated protein was removed by centrifugation and 2.0 ml. supernatant diluted to 25.0 ml. with distilled water; 1.0 ml. (equivalent to 800 μgm. original sugar) was then used for xylulose determination.

Experimental and Results

Since glutathione was used in attempts to reverse inhibitions caused by —SH reagents, it was necessary at the outset to test its effect on the isomerase activity. Glutathione had no significant effect upon the enzymatic conversion of D-xylose to D-xylulose by these preparations (see Table I). Controls were included in each experiment so that the necessary corrections could be made for the color contribution due to D-xylose.

The inhibitor studies will be presented in order of decreasing effectiveness of the inhibitors used.

p-Chloromercuribenzoate

This —SH inhibitor, originally introduced by Hellerman *et al.* (6), was found to be the most effective inhibitor of xylose isomerase. It completely inhibited the activity of the enzyme at a concentration of 2×10^{-4} *M*. The curve was extremely steep over a narrow range of concentration, i.e. very little inhibition was observed below 1×10^{-4} *M*. (Fig. 1). Addition of

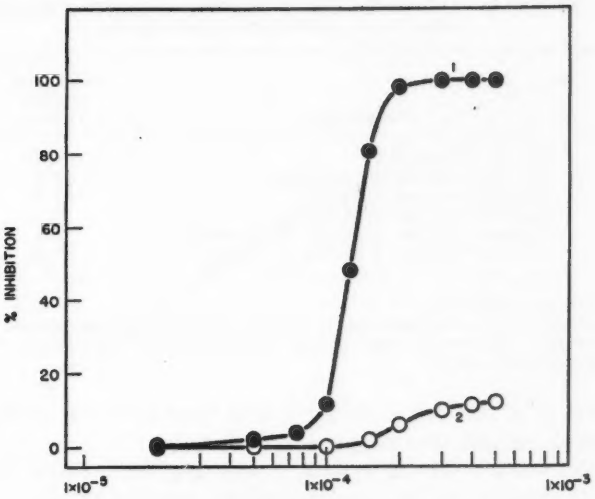

FIG. 1

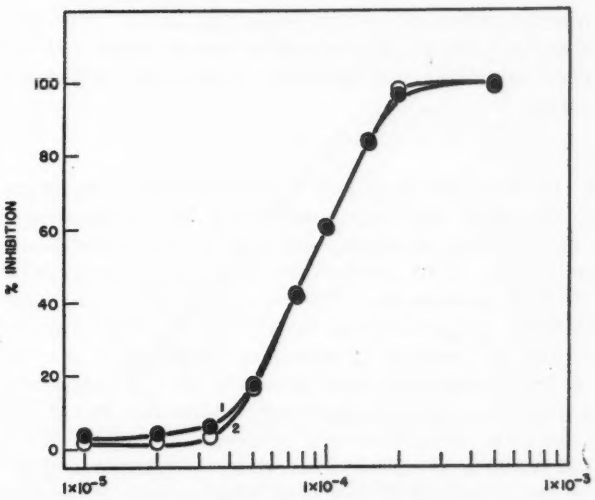

FIG. 2

FIG. 1. Inhibition of xylose isomerase by *p*-chloromercuribenzoate (*1*) and its reversal by glutathione (*2*).

FIG. 2. Inhibition of xylose isomerase by *p*-quinone (*1*) and the effect of glutathione (*2*).

TABLE I

EFFECTS OF GLUTATHIONE AND OF ALBUMIN ON XYLOSE ISOMERASE INHIBITED BY *p*-CHLOROMERCURIBENZOATE

Expt. no.	Components	D-Xylulose formed† (μgm.)	Inhibition (%)
1	Enzyme + xylose	63.9	—
2	Enzyme + xylose + glutathione	63.6	0.47
3	Enzyme + xylose + albumin	63.8	0.16
4	Enzyme + xylose + *p*CMB*	0.7	99.00
5	Enzyme + xylose + *p*CMB + glutathione	59.2	6.92
6	Enzyme + xylose + *p*CMB + albumin	57.9	9.24

* *p-Chloromercuribenzoate.*

† *Corrected for color contribution due to* D-*xylose, present in the assay system.*

Concentrations: Enzyme 10 mgm. protein, D-*xylose 0.18 M, glutathione* 10^{-3} *M, pCMB* 2×10^{-4} *M, albumin 1.25%.*

glutathione (5×10^{-3} *M*) produced a 95% reversal of the inhibition at the critical *p*-chloromercuribenzoate concentration.

Since this was a reversible system, it was possible to obtain the value for the affinity constant of the enzyme–inhibitor complex at 50% enzyme inhibition. At this point, the ratio of uncombined enzyme to enzyme–inhibitor complex is unity, thus $K_i = 1.27 \times 10^{-4}$ *M* (from Fig. 1).

The protective effect of protein mentioned earlier may be seen in the example given in Table I. At low enzyme concentration and in the presence of crystalline bovine plasma albumin, *p*-chloromercuribenzoate produced little inhibition.

p-Quinone

Quastel (16) first used *p*-quinone to inhibit urease. In recent years, considerable controversy has arisen concerning the mechanism of action of quinones as inhibitors of mitosis (9) and as antibacterial and antifungal substances (3, 10). More particularly, the question arose as to whether or not the effects of quinones on —SH enzymes would account entirely for the observed results. *p*-Quinone was found to be a potent inhibitor of xylose isomerase (Fig. 2), resulting in maximum inhibition at a concentration of 2×10^{-4} *M* and having little effect below 1×10^{-5} *M*. The curve was, thus, less steep than the one for *p*-chloromercuribenzoate but the concentration at which maximum inhibition was observed was identical for these two inhibitors at the same protein concentration. Glutathione (5×10^{-3} *M*) did not reverse the quinone inhibition, in striking contrast to the results obtained with *p*-chloromercuribenzoate.

N-Ethylmaleimide

This compound was found to form complexes with —SH containing substances by Friedmann *et al.* (5). Its rapid reaction suggested its use as a sulphydryl inhibitor. N-Ethylmaleimide was found to be much more effective

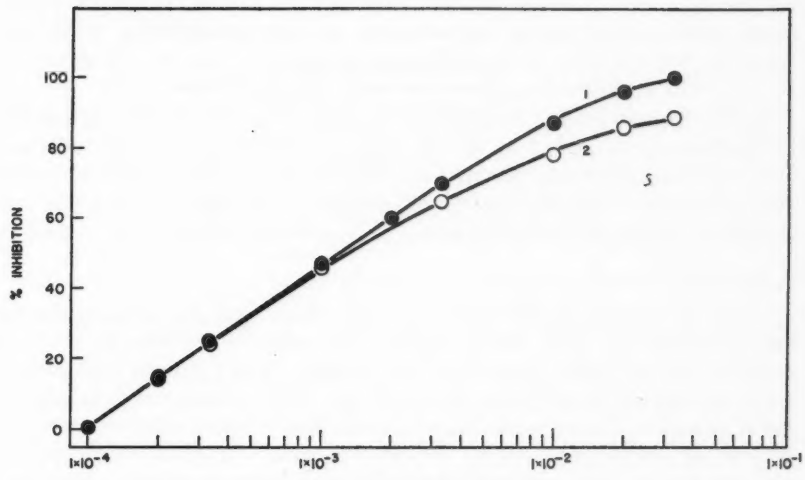

FIG. 3

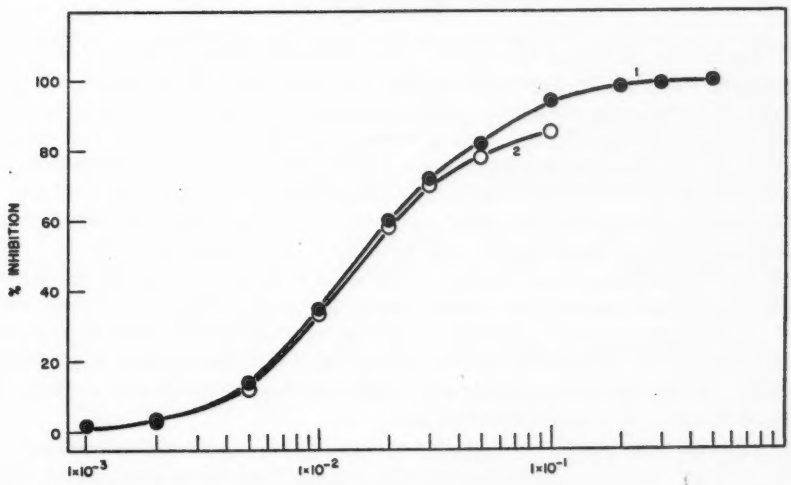

FIG. 4

FIG. 3. Inhibition of xylose isomerase by N-ethylmaleimide (*1*) and the effect of glutathione (*2*).

FIG. 4. Inhibition of xylose isomerase by iodoacetate (*1*) and the effect of glutathione (*2*).

than iodoacetate as an inhibitor of diphosphopyridine nucleotide-free triosephosphate dehydrogenase (17) but the order was reversed when the experiments were carried out in the presence of diphosphopyridine nucleotide. Neilands (15) found it to be ineffective with heart lactic dehydrogenase at 10^{-3} *M* while *p*-chloromercuribenzoate was a strong inhibitor at 10^{-4} *M*.

In the present study, N-ethylmaleimide was found to inhibit xylose isomerase completely at 3.3×10^{-2} *M* (Fig. 3). The type of inhibition curve obtained was quite different from those observed with *p*-chloromercuribenzoate and *p*-quinone. Inhibition was produced over a much wider range of concentrations in a more gradual manner. Glutathione (0.1 *M*) had little effect.

Iodoacetate

Since its early use in studies of muscular contraction (11), iodoacetate has been perhaps the most widely used −SH inhibitor. While some −SH enzymes are extremely sensitive to iodoacetate, others require high concentrations to produce significant effects (1, 2). Xylose isomerase falls into the latter category. As shown in Fig. 4, 0.3 *M* final concentration was required to produce complete inhibition, and below 2×10^{-3} *M* there was practically no effect at all. Glutathione (0.5 *M*) did not reverse the inhibition.

Discussion

The results presented show that xylose isomerase from *P. hydrophila* is an enzyme requiring intact −SH groups for activity. Glutathione reverses only the inhibition caused by *p*-chloromercuribenzoate but not that due to the other inhibitors used. The reversible combination of *p*-chloromercuribenzoate with the enzyme is similar to that found with other enzymes and the inability of glutathione to reverse the iodoacetate-inhibited system is further evidence in support of the view of Barron and Singer (2) that iodoacetate may inhibit by a mechanism other than by simple combination with −SH groups. The slope of the inhibition curve as well as the high concentration of iodoacetate required to inhibit might be used in support of this idea. A recent observation (17) that the reactivity of −SH inhibitors with proteins may be largely determined by the properties of adjacent groups is pertinent here. This suggestion might also apply to N-ethylmaleimide under the experimental conditions described here.

The fact that quinone completely inhibits xylose isomerase irreversibly at exactly the same concentration (for the same amount of protein) as does *p*-chloromercuribenzoate is interesting. It is possible that these two inhibitors combine with the same groups on the enzyme, but that, in addition, quinone also combines with adjacent groups.

While this paper was in preparation, a report appeared (18) showing that xylose isomerase is also found in *Pasteurella pestis*. Several properties were described which appear to be different from the *P. hydrophila* enzyme. The *P. pestis* enzyme was also inhibited reversibly by *p*-chloromercuribenzoate

and reactivated with cysteine. A strict comparison with the data given in the present paper is not possible, since few values are available for the various —SH inhibitors used.

Acknowledgment

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References

1. BAILEY, K. and WEBB, E. C. Purification of yeast hexokinase and its reaction with $\beta\beta'$-dichlorodiethyl sulphide. Biochem. J. 42 : 60–68. 1948.
2. BARRON, E. S. G. and SINGER, T. P. Sulphydryl enzymes in carbohydrate metabolism. J. Biol. Chem. 157 : 221–240. 1945.
3. COLWELL, C. A. and MCCALL, M. Studies on the mechanism of antibacterial action of 2-methyl-1,4-naphthoquinone. Science, 101 : 592–594. 1945.
4. DISCHE, Z. and BORENFREUND, E. A new spectrophotometric method for the detection and determination of keto sugars and trioses. J. Biol. Chem. 192 : 583–587. 1951.
5. FRIEDMANN, E., MARRIAN, D. H., and SIMON-REUSS, I. Antimitotic action of maleimide and related substances. Brit. J. Pharmacol. 4 : 105–108. 1949.
6. HELLERMAN, L., CHINARD, F. P., and DEITZ, V. R. Protein sulfhydryl groups and the reversible inactivation of the enzyme urease. J. Biol. Chem. 147 : 443–462. 1943.
7. HOCHSTER, R. M. and WATSON, R. W. Xylose isomerase. J. Am. Chem. Soc. 75 : 3284. 1953.
8. HOCHSTER, R. M. and WATSON, R. W. Enzymatic isomerization of D-xylose to D-xylulose. Arch. Biochem. Biophys. 48 : 120–129. 1954.
9. HOFFMANN-OSTENHOF, O. Die Biochemie der Chinone. Experientia, 3 : 176–184. 1947.
10. HOFFMANN-OSTENHOF, O. Mechanism of the antibiotic action of certain quinones. Science, 105 : 549–550. 1947.
11. LUNDSGAARD, E. Untersuchungen über Muskelkontraktionen ohne Milchsäurebildung. Biochem. Z. 217 : 162–177. 1929.
12. MCILWAIN, H. Preparation of cell-free bacterial extracts with powdered alumina. J. Gen. Microbiol. 2 : 288–291. 1948.
13. MCKENZIE, H. A. and WALLACE, H. R. The Kjeldahl determination of nitrogen: a critical study of digestion conditions—temperature, catalyst, and oxidizing agent. Aust. J. Chem. 7 : 55–70. 1954.
14. MITSUHASHI, S. and LAMPEN, J. O. Conversion of D-xylose to D-xylulose in extracts of *Lactobacillus pentosus*. J. Biol. Chem. 204 : 1011–1018. 1953.
15. NEILANDS, J. B. Studies on lactic dehydrogenase of heart. III. Action of inhibitors. J. Biol. Chem. 208 : 225–230. 1954.
16. QUASTEL, J. H. The action of polyhydric phenols on urease; the influence of thiol compounds. Biochem. J. 27 : 1116–1122. 1933.
17. RACKER, E. Mechanism of action and properties of pyridine nucleotide-linked enzymes. Physiol. Revs. 35 : 1–56. 1955.
18. SLEIN, M. W. Xylose isomerase from *Pasteurella pestis*, Strain A-1122. J. Am. Chem. Soc. 77 : 1663–1667. 1955.

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